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14. ABSTRACT: During breast cancer development, increased presence of leukocytes in stroma parallels disease progression; however, functional significance of leukocytes in regulating pro- versus anti-tumor immunity in the breast remains poorly understood. Utilizing the MMTV-PyMT model of mammary carcinogenesis, we have demonstrated that cathepsin C-expressing macrophages and IL-4-expressing $CD4^+$ T cells indirectly promote invasion and subsequent metastasis of mammary adenocarcinomas. $CD4^+$ T cells regulate the phenotype and effector function of macrophages that in turn enhance metastasis through activation of EGF receptor signaling in malignant epithelial cells. Together, these data indicate that anti-tumor acquired immune programs can be usurped in pro-tumor microenvironments and instead promote malignancy by engaging cellular components of the innate immune system functionally involved in regulating epithelial cell behavior. Based on this data, we revealed a unique immune signature that predicts overall survival of women with breast cancer. Moreover, we have revealed that transient blockade of Alk5 enhances delivery of high molecular weight compounds into mammary tumors. We will employ this capability and evaluate cell-based delivery systems and targeted-iron oxide imaging compounds to non-invasively evaluate "inflammation" in mammary carcinomas, to not only target leukocytes to minimize their tumor-promoting activities, but also to develop imaging modalities to predict outcome for patients and help guide therapy.

15. SUBJECT TERMS
breast cancer, mouse models, organotypic culture, inflammation, proteases, cathepsin protease, immune cells, leukocytes

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I. INTRODUCTION:

The association of immune cells and cancer has been known for over a century[1]. Individuals prone to chronic inflammatory diseases have a greatly enhanced risk for cancer development and cancer patients with malignant tissues containing infiltrates of T_H2-polarized immune cells tend to have a worse clinical prognosis[2]. Epidemiological studies reported that inhibiting chronic inflammation in patients with pre-malignant disease or patients predisposed to cancer development has demonstrated chemopreventative potential[3]. These studies revealed that long-term usage of anti-inflammatory drugs, e.g. aspirin and selective cyclooxygenase (COX)-2 inhibitors significantly reduces cancer (breast, prostate, colon, renal, lung) risk[4].

Because of their enormous plasticity and capacity to produce a cytokines, chemokines, metallo- serine and cysteine proteases, reactive oxygen species (ROS), histamine and other bioactive mediators, chronically activated immune cells are key modulators of cell survival as well as regulators of ECM metabolism. Thus, physiologic processes necessary for tumor development, i.e., cell survival, tissue remodeling, and angiogenesis, are regulated by immune cells. This is exemplified by positive correlations between numbers of myeloid cells infiltrating human tumors with number of blood vessels[5], and experimental findings in mouse models where attenuating immune cell infiltration reduces angiogenesis and primary tumor development[6-8].

We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. To address these issues, we have taken several approaches to investigate mechanisms involved in: *i.* induction and maintenance of inflammatory microenvironments in premalignant and malignant tissues, *ii.* Assess the role of leukocyte and their proteases as regulators of cancer development, and *iii.* development of novel non-invasive imaging methodologies to monitor inflammation and/or deliver therapeutics to carcinoma cells. Our studies are designed to test the hypothesis that *inflammation* is a critical parameter of neoplastic development and therefore represents an efficacious target for anti-cancer therapies.

II. RESEARCH ACCOMPLISHMENTS BODY:

Task 1. Define the profile and proteolytic contribution of leukocytes in human breast cancer and in transgenic mouse models of mammary carcinogenesis.

a. Elucidate the spectrum of $CD45^+$ cells in normal and neoplastic human breast tissues. Months 1-12

COMPLETE: Results were reported in the *2007 Annual Progress Report* and have now been published[9,10]

b. Elucidate the spectrum of $CD45^+$ cells in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis. Months 1-24

COMPLETE: Results were reported in the *2008 Annual Progress Report* and have now been published[10]. Also see below for Figure 3 and 4.

c. Develop a profile of proteolytic activities in normal and neoplastic human breast tissues. Months 1-12

COMPLETE: Results were reported in the *2007 Annual Progress Report* and have now been published[11]

d. Develop a profile of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis. Months 1-24

COMPLETE: Results were reported in the *2007 Annual Progress Report*

e. Determine cellular origins of proteolytic activities in normal and neoplastic human breast tissues. (initial projection: Months 1-12; revised projection: Months 24-36)

Since our experimental animal studies have revealed a functional role for the cysteine protease cathepsin C as a mediator of mammary carcinoma metastasis, we are focusing on characterizing cathepsin C expression in human breast tissues. Since our studies assessing the functional significance of CD4+ T cells as mediators of breast cancer metastasis have been quite successful (see below), we have focused on these analyses in the past years at the expense of expanding our studies of leukocyte proteases that will now become more of a priority. Thus far, we have assessed cathepsin C expression in a series of human invasive breast cancers and colocalized cathepsin C expression to CD68+ cells predominantly, indicating that macrophages express

cathepsin C (**Figure 1**). With that in our other studies we have found that macrophages regulate breast cancer metastasis by modulating the epidermal growth factor-activated signaling pathway[10], current investigations are aimed at understanding if macrophage-cathepsin C in part regulates this pathway downstream of interleukin (IL)-4[10].

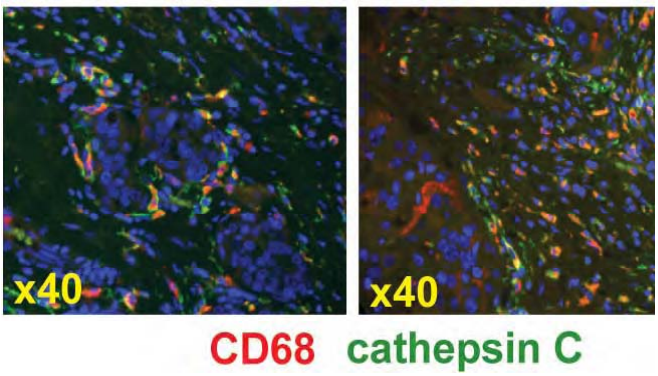


Figure 1: Colocalization of cathepsin C (green) in CD68-positive macrophages (red) in human breast adenocarcinomas

f. Determine cellular origins of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis. Months 1-24

Based upon the 7-fold increased mRNA expression of *cathepsin C* revealed by gene expression profiling analysis in tissues from MMTV-PymT mice **(reported in our 2007 Annual Progress Report**), we examined tissue sections for cathepsin C protein expression and found that infiltrating immune cells are the predominant source of the enzyme (**reported in our 2008 Annual Progress Report**). By utilizing double immunofluorescence staining, we have revealed that macrophages are the primary source of cathepsin C amongst the CD45+ cells infiltrating primary mammary carcinomas and metastatic lungs (**reported in our 2008 Annual Progress Report; Figure 2)**. Based on this compelling data, we are undertaking in vivo studies to assess the functional significance of cathepsin C in MMTV-PyMT mice currently. Our preliminary data is reported below.

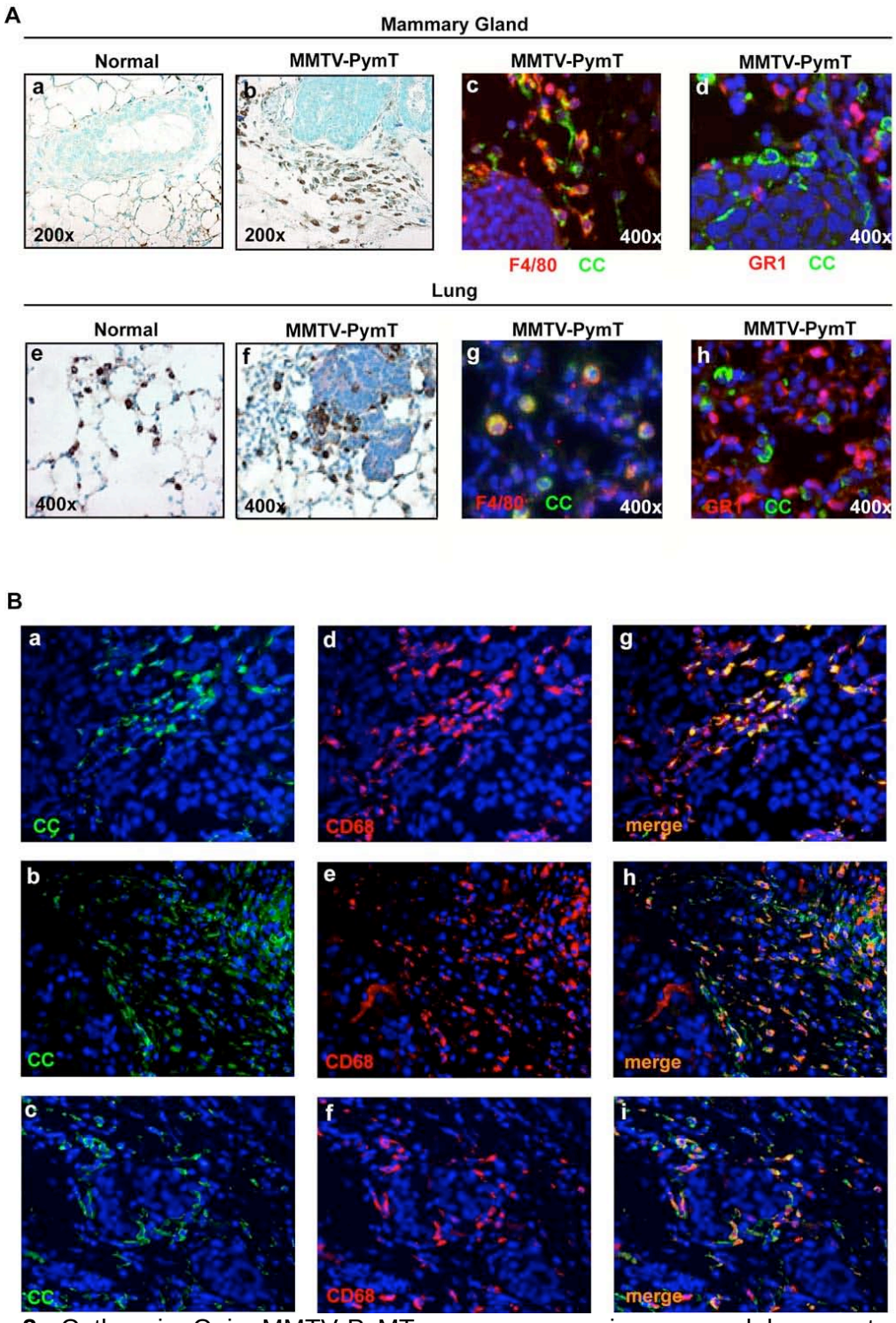


Figure 2: Cathepsin C in MMTV-PyMT mammary carcinomas and lung metastases. A) Cathepsin C protein expression is detectable by immuno-detection in normal nontransgenic mammary (a) and lung (e) tissue (brown staining) and in MMTV-PymT mammary (b) and lung (f) tissue at d110. Dual immuno-fluorescent staining for cathepsin C (green) and either F4/80 (panel c and g, red staining) or GR-1 (panel d and h, red staining) localizes cathepsin C expression to F4/80+ positive macrophages in malignant mammary tissue and metastatic lungs. B) Dual immunofluorescent staining for cathepsin C (green, a-c) and CD68 (red, d-f) localizes cathepsin C expression to CD68 positive macrophages in merged images (g-i) from human breast cancer tissue sections

g. Compare immune cell infiltrations in mouse and human normal and neoplastic tissues. Identify candidate cell types for further study. Months 1-24

COMPLETE: Comparative results were reported in the *2008 Annual Progress Report*, quantitation of salient immune cell populations is now provided below (**Figure 3**).

As observed in several types of solid tumors, human breast adenocarcinomas are characterized by infiltration of both innate and adaptive immune cells[10,12]. Immunohistochemical (IHC) detection of $CD68^+$ myeloid cells (macrophages), $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells in human breast cancer reveals an increase in each cell type paralleling cancer development (**Figure 3**)[10]. High percentages of $CD4^+$ T cells in primary breast cancers positively correlate with markers of tumor stage, including metastatic spread to sentinel LNs and increased primary tumor size[13]. Perhaps more significant, the ratio of $CD4^+$ to $CD8^+$ T cells or T_H2 to T_H1 cells present in primary tumors, where $CD4^+$ or T_H2 cells are more frequent than $CD8^+$ or T_H1 cells, correlates with LN metastasis and reduced overall patient survival[13]. More recently, unsupervised expression profiling from breast cancer-associated stroma revealed a gene signature predictive of good prognostic outcome (>98%, 5 year survival) functionally enriched for elements of a T_H1-type immune response, including genes suggestive of CTL and NK cell activity[14]. Conversely, high levels of $FOXP3^+$ T_{reg} cells predict diminished relapse-free and overall patient survival[15]. The interpretation based upon these clinical studies is that the type of $CD4^+$ effector T cell response elicited in an emergent breast cancer may in part determine malignant and metastatic potential. Based upon these compelling associations, and our experimental findings in PyMT mice[10], we predicted that the composition and *signature* of leukocytes that infiltrate human breast cancer might contain predictive power and correlate with overall survival. As such, we quantitatively examined a human tissue microarray (TMA) containing 198 invasive breast cancers tissues for relative presence of $CD4^+$ and $CD8^+$ T cells, and $CD68^+$ macrophages and found that 5-year overall survival was significantly diminished when malignant tissue contained the immune signature $CD4^{hi}CD8^{lo}CD68^{hi}$ as opposed to $CD4^{lo}CD8^{hi}CD68^{lo}$ and represented an independent prognostic indicator of overall survival based on multivariate cox-regression analysis (**Figure 4**). Based upon this compelling date, we are evaluating a second larger "validation cohort" consisting of 498 consecutive patients with primary invasive breast cancer and median follow-up time to first breast cancer event of 128 months. This larger cohort contains significant representation of invasive disease representing

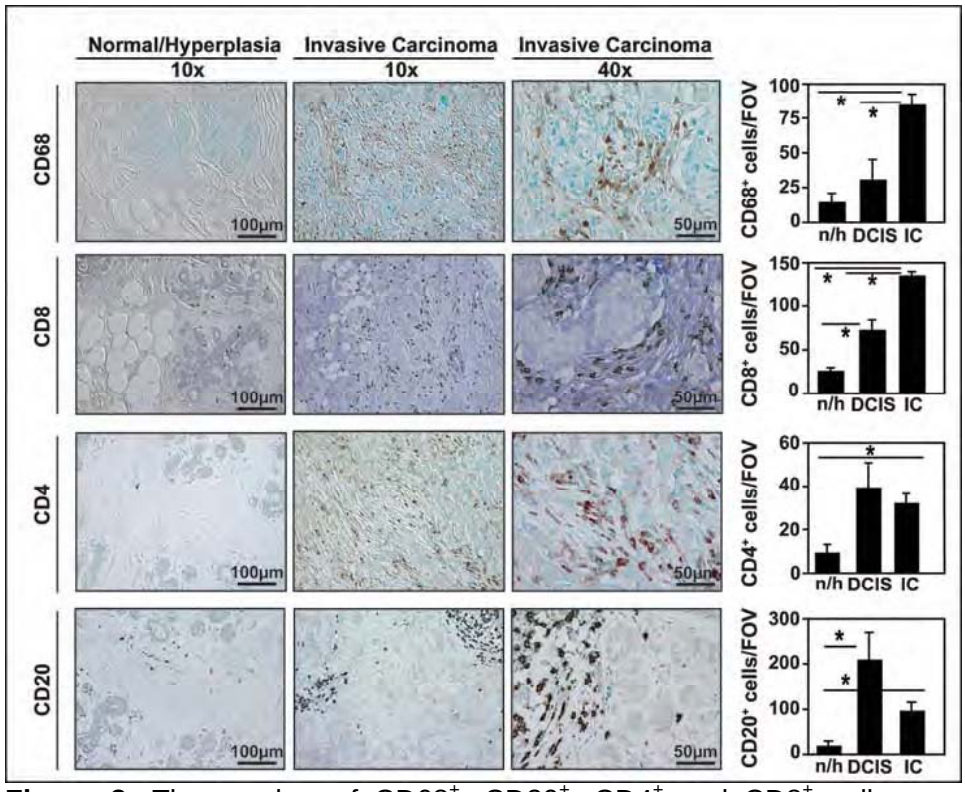
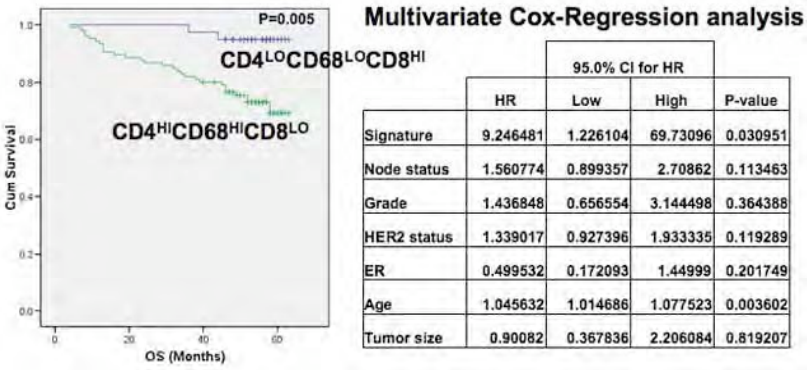


Figure 3: The number of $CD68^+$, $CD20^+$, $CD4^+$ and $CD8^+$ cells was analyzed in patient samples of normal/hyperplastic breast tissue (n/h; n=9), ductal carcinoma *in situ* (DCIS; n=6) and invasive ductal carcinomas (IC; n=150) using tissue micro-arrays. Representative 10x and 40x images are shown and the average number of positive cells as depicted reflects the mean number of cells in each disease stage, evaluated by counting all high power fields (20x) per tissue section (1.1 mm)/2 sections/patient. * $p<0.05$ by Mann-Whitney.

basal, luminal and triple-negative breast cancer; thus, we will evaluate if the predictive signature stratifies any one histological subtype with preference. We have already begun preliminary investigation with triple-negative breast cancer metastases to assess infiltration by various leukocytes (**Figure 5**).



Multivariate Cox-Regression analysis

		95.0% CI for HR		
	HR	Low	High	P-value
Signature	9.246481	1.226104	69.73096	0.030951
Node status	1.560774	0.899357	2.70862	0.113463
Grade	1.436848	0.656554	3.144498	0.364388
HER2 status	1.339017	0.927396	1.933335	0.119289
ER	0.499532	0.172093	1.44999	0.201749
Age	1.045632	1.014686	1.077523	0.003602
Tumor size	0.90082	0.367836	2.206084	0.819207

Figure 4: Immune signature predicts overall survival. Quantitative evaluation of $CD4^+$, $CD8^+$ and $CD68^+$ cells infiltrates in 198 invasive breast cancers, as evaluated using Aperio image analysis, and correlated with 5-yr overall survival (OS).

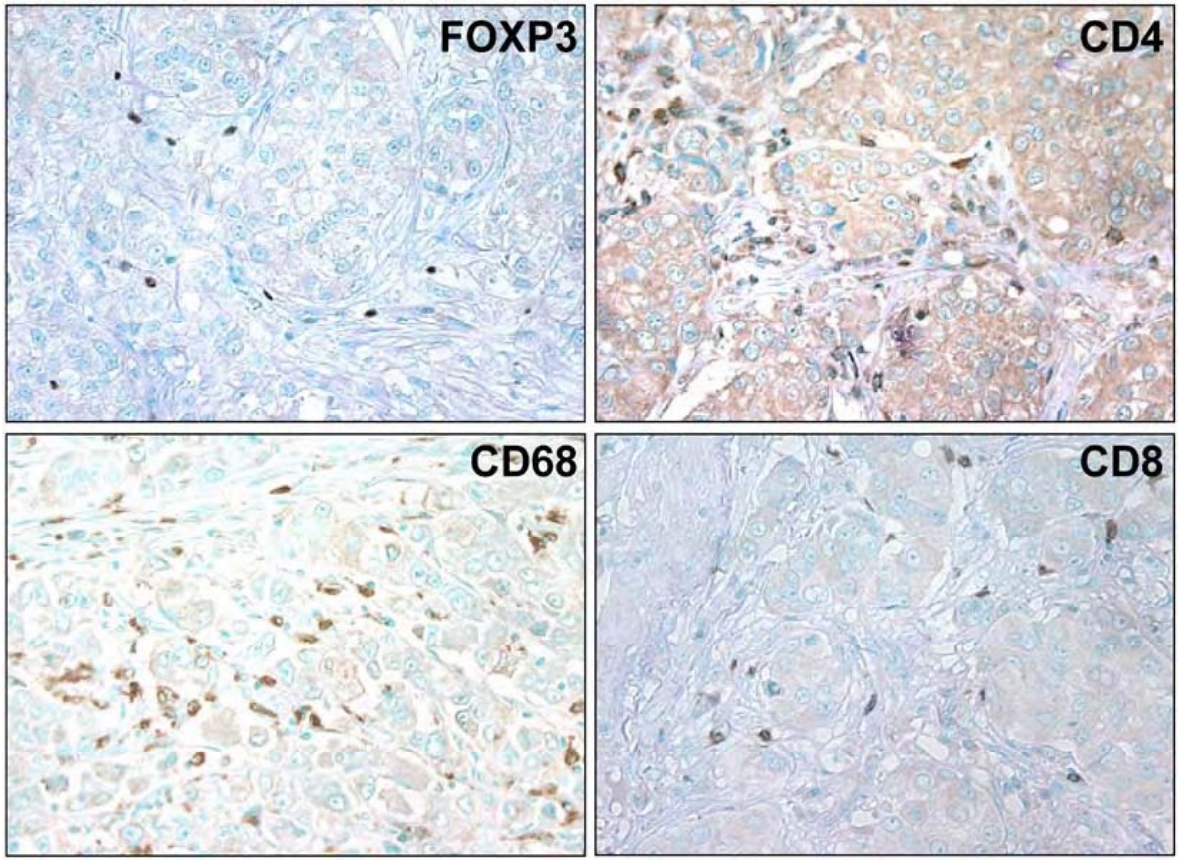


Figure 5: Immunodetection of CD68-positive macrophages, FOXP3-, CD4- and CD8-positive T cells in a lung metastasis from a woman with triple-negative breast cancer. Magnification shown for all is 40X.

In addition, we are evaluating a retrospective cohort containing 207 high-risk DCIS samples from patients with a median follow-up of 155 months and a 24% local recurrence rate to reveal if the $CD4^{hi}CD8^{lo}CD68^{hi}$ profile stratifies the cohort. Thus, for a woman with her initial diagnosis, where information is power, we anticipate that recognition of this immune signature will guide decisions on course of therapy such that the 20-30% of women diagnosed with breast cancer whose initial prognostic markers are favorable based on tumor size, tumor grade, and LN status, but still go on to develop metastatic disease within 10 years will be revealed and instead receive aggressive therapy (cytotoxic-, immuno-, and/or targeted-therapy) to minimize risk of progression and recurrence.

As an extension to these studies, we have spent considerable effort over the past year optimizing Fluorescent Activated Cell Sorting of tissues to enable simultaneous quantitative evaluation of multiple lineage-selective markers in complex tissues of either mouse or human origin. Results from one such representative FACS of a murine mammary gland is shown in **Figure 6**, where we have used 12 color-FACS to evaluate lineage expression representing 8 distinct immune cell types.

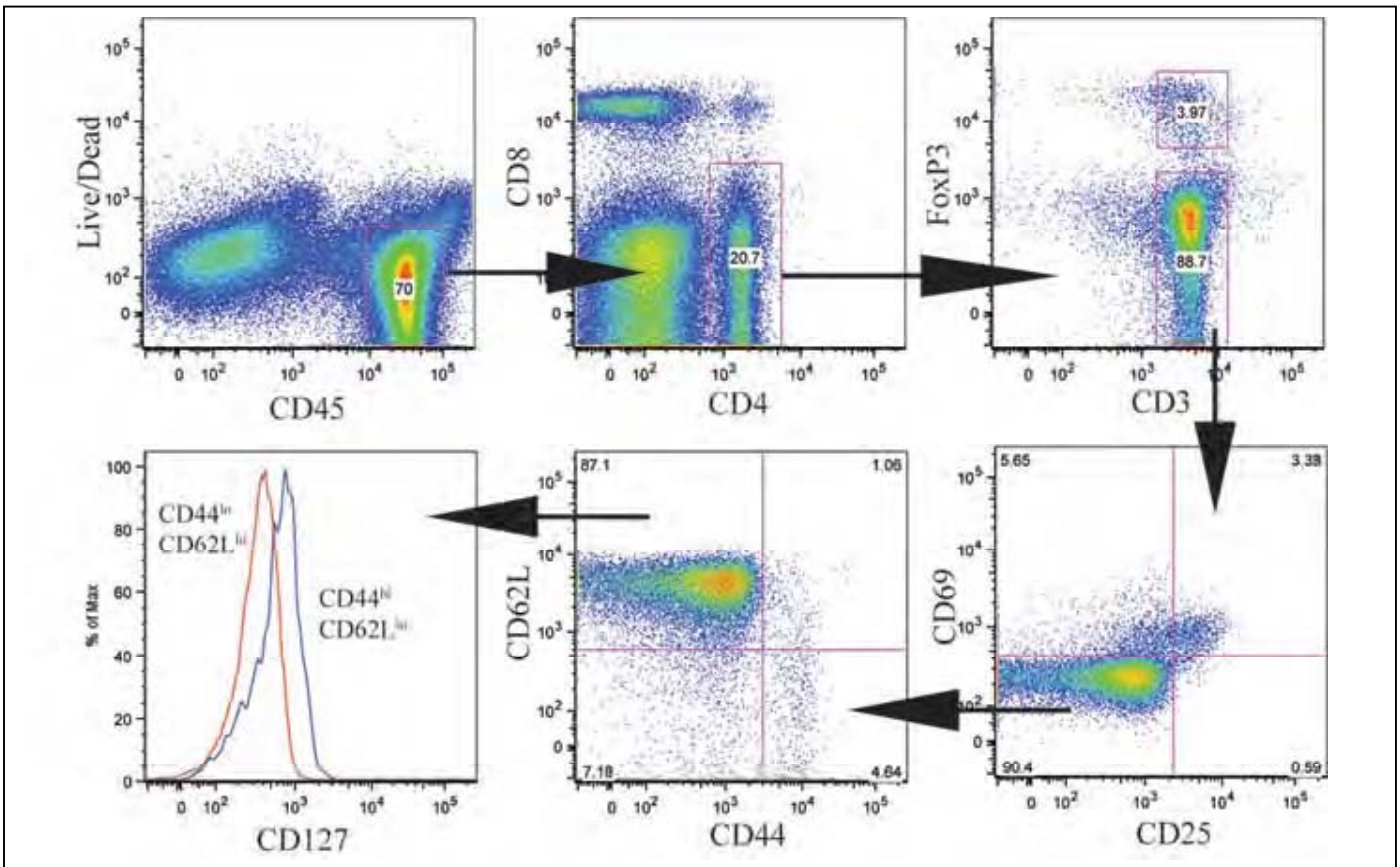


Figure 6: Analysis of splenic T cell populations in FvBN mice using 12-color flow cytometry. A single cell suspension of splenocytes were Fc blocked with 2.4G2, then incubated with 10 primary conjugated antibodies (CD45, B220, CD3, CD4, CD8, CD25, CD44, CD62L, CD69, CD127) for cell surface antigen staining, along with a Live/Dead stain for exclusion of dead cells. Cells were then fixed, permeabilized, and stained with a FoxP3 primary conjugated antibody. Data was collected using a BD LSRII Flow Cytometer and analyzed using FlowJo software

h. Compare proteolytic activities in mouse and human normal and neoplastic tissues based. Identify candidate protease activities for further study. (initial projection: Months 1-24; revised projection Month 24-48

As of month 36, these comparative analyses have not been initiated. We have recently developed an activity probe for cathepsin C[16]; however, obtaining unfixed, fresh human breast caner tissue has proven more difficult than initially thought. To resolve this issue, we recently initiated collaboration with Dr. Susan Love (Dr. Susan Love Medical Research Foundation) and Dr. Lisa Bailey (Alta Bates Hospital) to obtain freshly resected human breast cancer tissue via the UCSF Tissue Core and our existing CHR approvals. As we begin receiving these tissues, we will begin assessing cathepsin C activity and anticipate completion of this aim over the next 24 months.

Task 2. Validate target molecules and/or specific immune cell types in biological assays and in animal models of mammary carcinogenesis.

a. Establish 3-dimensional mammary epithelial organotypic cell culture model system cultured on basement membrane. Months 1-9

COMPLETE: Results were reported in the *2007 Annual Progress Report* and have been published[10]

b. Establish routine isolation and short-term culture conditions for mouse leukocytes. Months 1-9

COMPLETE: Results were reported in the *2007 Annual Progress Report* and have now been published[10]

c. Establish 3-dimensional organotypic co-culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic cell types. Months 6-12

COMPLETE: Results were reported in the 2007 and *2008 Annual Progress Reports* and have now been published[10]

d. Establish 3-dimensional organotypic culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic-derived proteases. Months 6-12

COMPLETE: Results were reported in the *2007 Annual Progress Report*. In the next funding cycle, based on compelling data from in vivo studies with cathepsin C-deficient/PymT mice, we will return to these assays to evaluate paracrine regulation between cathepsin C-positive vs cathepsin C-deficeint leukocytes in regulating aspects of mammary epithelial cell physiology.

e. Analyze role of leukocytes in regulating mammary epithelial morphogenesis, proliferation, cell death, apicobasal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in normal and oncogene-expressing 3-dimensional acini cultured on basement membrane. Months 12-24

COMPLETE: Results were reported in the *2008 Annual Progress Report* and have now been published[10]

f. Analyze role of leukocyte-derived proteases in regulating mammary epithelial morphogenesis, proliferation, cell death, apicobasal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in normal and oncogene-expressing 3-dimensional acini cultured on basement membrane. Months 12-36

These assays have now been established as reported in our 2007 and 2008 progress report; however, due to our focus on evaluating functional roles for leukocytes as regulators of mammary carcinogenesis, studies on protease function in vitro were delayed pending completion of our current leukocyte studies. Thus, over the next funding period, ex vivo assessment of cathepsin C as a paracrine mediator will be a priority.

g. Analyze role of candidate leukocytes by crossing mice deficient in or modified such that individual or classes of leukocytes (identified in Task 1) are deficient with transgenic mice prone to development of mammary adenocarcinoma. Months 1-48

We are happy to report that the main focus of our work in this area is now in press at Cancer Cell[10]. The manuscript can also be found in Appendix B. Below reflects a brief summary of the work and plans for ongoing experiments.

During breast cancer development, increased presence of leukocytes in neoplastic stroma parallels disease progression; however, functional significance of leukocytes in regulating pro-tumor versus anti-tumor immunity in the breast remains poorly understood. Macrophages, which are part of the innate immune response, can mediate metastasis of malignant mammary epithelial cells (MECs) by secreting epidermal growth factor (EGF). However, adaptive immune cells, such as $CD4^+$ and $CD8^+$ T cells, that regulate the function of innate immune cells are also present. What is the function of these cells in breast cancer? We have examined the development of breast cancers driven by the Polyoma middle T (PyMT) antigen under the control of the mouse mammary tumour virus (MMTV) promoter in mice with dysfunctional adaptive immune systems[10]. We found that these mice showed no changes in primary tumour growth and angiogenesis, but mice lacking $CD4^+$ T cells, or mice with established tumours that were then depleted of $CD4^+$ T cells had reduced levels of pulmonary metastases, as well as fewer circulating tumour cells[10].

Given the association of macrophages with breast cancer metastasis, we asked whether $CD4^+$ T cells could affect macrophage function in breast tumours. Although macrophages were still present in breast tumours in MMTV–*PyMT*;*Cd4* $^{-/-}$ mice, analysis of their activation status indicated that they had an M1 phenotype, whereas those in mice with $CD4^+$ T cells were of an M2 phenotype[10]. M2 macrophages, unlike M1 macrophages express pro-angiogenic, pro-tissue remodelling cytokines in response to T helper 2 cytokines, such as interleukin 4 (IL-4), IL-10 and IL-13. Indeed, $CD4^+$ T cells isolated from the mouse mammary tumours expressed these cytokines.

To show that $CD4^+$ T cells and the cytokines they produce were the driving force for metastasis, we cultured MECs from MMTV–PyMT mice in 3D organoid culture and then added tumour associated macrophages and $CD4^+$ T cells isolated from MMTV–PyMT mice. Only under these conditions did malignant MECs grow invasively. Moreover, *MMTV–PyMT;IL-4Ra*$^{-/-}$ mice also showed reduced levels of pulmonary metastases, suggesting that this $CD4^+$ T cell-expressed cytokine is crucial. How does IL-4 affect the macrophages? IL-4 significantly increased the expression of EGF by the macrophages and inhibition of the EGF–EGFR pathway suppressed the invasive growth of malignant MECs in the 3D co-culture assay[10].

Therefore, the innate and adaptive immune responses collaborate to induce pulmonary metastases in mice with mammary tumours. Thus, we have demonstrated a tumor-promoting role for T_H2-$CD4^+$ T lymphocytes that elicit pro-tumor, as opposed to cytotoxic bioactivities of tumor-associated macrophages and enhancement of pro-metastatic epidermal growth factor receptor signaling programs in malignant mammary epithelial cells. This work reveals a novel pro-tumor regulatory program involving components of the acquired and cellular immune systems that effectively collaborate to promote pulmonary metastasis of mammary adenocarcinomas, and identifies new cellular targets, namely $CD4^+$ T effector cells and IL-4 for anti-cancer therapy.

IL-4 induces macrophage EGF expression and EGFR/CSF1R-dependent invasion and metastasis: To identify the soluble mediators released by TAMs following their activation by $CD4^+$ T cells and IL-4 that induce MEC invasion and metastasis, we evaluated TAM expression of several growth factors associated with epithelial cell invasion and found that TAMs isolated from CD4-proficient/PyMT mice expressed higher levels of EGF and TGFβ mRNA[10], as compared to TAMs from $CD4^+$ T cell-deficient/PyMT mice (**Figure 7A**). To determine if enhanced EGF expression by TAMs was directly due to IL-4 exposure, we evaluated TAM EGF mRNA expression following brief exposure to IL-4, CSF-1, IL-4 plus CSF-1, as compared to pMEC conditioned medium (CM) alone, and found that IL-4 significantly enhanced EGF mRNA expression, but only in the presence of CSF-1 or pMEC CM (**Figure 7A**). An outstanding question to answer regarding this data has to do with the autocrine relationship between IL-4, IL-13 and their cognate receptors. We do not know if EGF mRNA can be induced to the same levels following combined IL-4 and IL-13 exposure or if instead high-level expression is CSF-1-dependent.

To establish if activation of EGFR-mediated signaling in MECs was necessary for TAM-induced invasion, we evaluated EGFR blockade using the 3D co-culture assay (**Fig 8**) and found that IL-4-regulated TAM-dependent pMEC invasion was significantly diminished in the presence of EGFR tyrosine kinase inhibitors PD153035 or BIBX1382 (**Fig 8**). Analogously, we evaluated if EGFR signaling *in vivo* regulated dissemination

of malignant cells into circulation - late-stage (d110) PyMT mice were treated with PD153035 (25 mg/kg) for 5 hours resulting in a significant decrease in number of circulating carcinoma cells present in PB (**Figure 7B**).

To evaluate the role of CSF1R signaling as a mediator of invasive and/or metastatic potential of mammary tumors in PyMT mice, we assessed CSF1R blockade first using the 3D co-culture assay and found that similar to EGFR blockade, IL-4-regulated TAM-dependent pMEC invasion was significantly diminished in the presence of CSF1R tyrosine kinase inhibitors PLX3397 and PLX5622 (**Figure 7B**). In addition, late-stage (d110) PyMT mice were treated with PLX3397 and PLX5622 (40 mg/kg) for 5 hours followed by assessment of circulating carcinoma cells in PB that were significantly decreased (**Figure 7B**). Taken together, these data indicate that in response to $CD4^+$ T cell-derived IL-4, M2 effector bioactivity is enhanced in TAMs that results in enhanced production of EGF and activation of a pro-invasive feed forward loop involving CSF1R on MECs[10]. In Aim 1 and 2, we will address the therapeutic potential of EGFR- and CSF1-blockade as combinatorial therapy with either IL-4/13 ligand or receptor blockade to reveal the most efficacious strategy to minimize metastasis and neutralize T_H2 immunity in the primary and metastatic microenvironments.

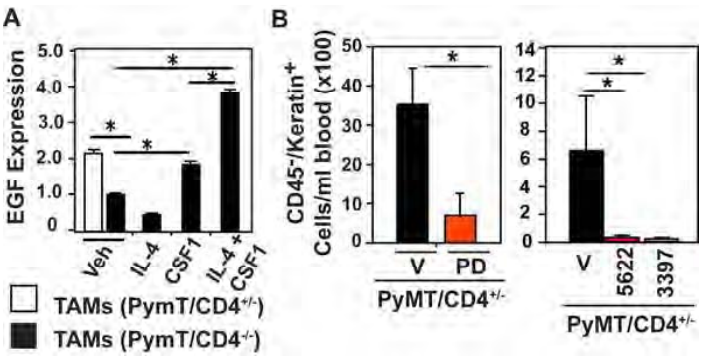


Figure 7: IL-4 and CSF1 regulate EGF expression and peripheral blood dissemination of tumors. A) EGF mRNA expression in freshly isolated TAMs, and TAMs briefly exposed to IL-4, CSF-1 or IL-4+CSF1. **B)** Presence of circulating carcinoma cells in PB of PyMT mice following 5-hr of treatment with an EGFR inhibitor PD153035 (left graph; PD, 25 mg/kg), or CSF1R inhibitors PLX3397 (3397) or PLX5622 (5622) (right graph; 40 mg/kg). $p<0.05$.

Determining cellular sources of IL-4/13 during mammary carcinogenesis: To identify the spatial, temporal and cellular characteristics of IL-4 and IL-13 expression during mammary carcinogenesis *in vivo*, we have employed IL-4 reporter mice that express enhanced green fluorescent protein (EGFP) in the *IL-4* locus linked via a viral IRES element, i.e., 4get mice[17], intercrossed with PyMT mice. Our initial evaluation of late-stage mammary adenocarcinomas in these bigenic mice reveals $CD45^+$IL-4/GFP^+ cells in tumor stroma (**Figure 8**), and by flow cytometry, reveals identity of these cells to be predominantly $CD4^+$ T cells (data not shown). Similarly, mice expressing bicistronic IL-13/EGFP (13get mice) have been constructed and are currently being evaluated (R. Locksley, personal communication) and intercrossed with PyMT mice (data not shown). 4get and 13get mice will reveal cellular sources of ligands during primary tumor development and metastasis, as well as *in vivo* indicators for when during progression the T_H2 microenvironment in primary tumors and metastases is established. This will be important information to elucidate as it will guide vaccination strategies in Aim 3, and provide a means to gauge success of neutralizing the T_H2 microenvironment following IL-4/13- and/or IL-4/13R-blockade (Aim 1-2) and durability of those responses.

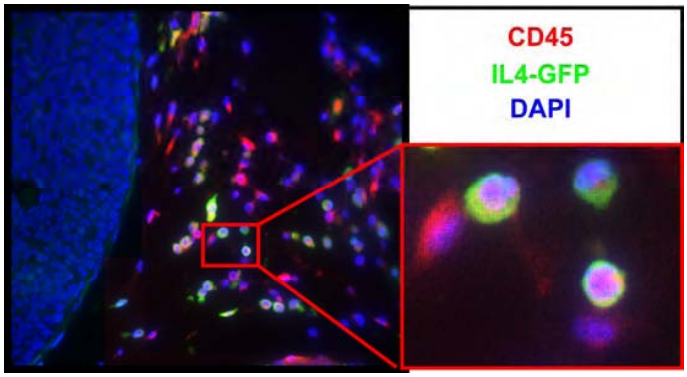


Figure 8. Localizing IL-4 expressing cells using IL4-GFP reporter 4get mice. IF detection of GFP^+ (green) and $CD45^+$ (Red) cells in mammary tumor tissue from d90 4get/PyMT mice, counterstained with DAPI (blue).

Dendritic cells in primary and metastatic tumors: Dendritic cells (DCs) have the capacity to significantly shape immune responses to cancer by both regulating innate immunity through production of cytokines and chemokines, as well as regulating pro- vs anti-tumor T cell responses by antigen presentation. Because individual DC subsets can reflect altered bioactivities, we assessed changes in DC populations in primary mammary adenocarcinomas versus pulmonary metastases by polychromatic flow cytometry **(Figure 9).** In both

primary tumors and secondary pulmonary metastases, we found variances in DC subset infiltration with significant accumulations of mDCs (**Figure 9A**) similar to studies in Oncohumice harboring human breast cancers and in human breast cancer tissues[18]. Since the tumor microenvironment clearly regulates DC subtype recruitment/maturation, antigen presentation by DCs to $CD4^+$ and/or $CD8^+$ T lymphocytes is also regulated by the microenvironment where it exerts a pleotropic role in shaping T-effector cell response (aka T_H1 vs T_H2), metastatic potential of primary tumors and likely response to immuno-, targeted-, and cytotoxic-therapy. A major goal of the P01 application and P5 will be to determine if these infiltrating mDCs are responsible for "driving" protumor T_H2 immunity, and revealing the most efficacious approach to diminish this process and instead promote a T_H1 immune microenvironment where durable anti-tumor responses are fostered.

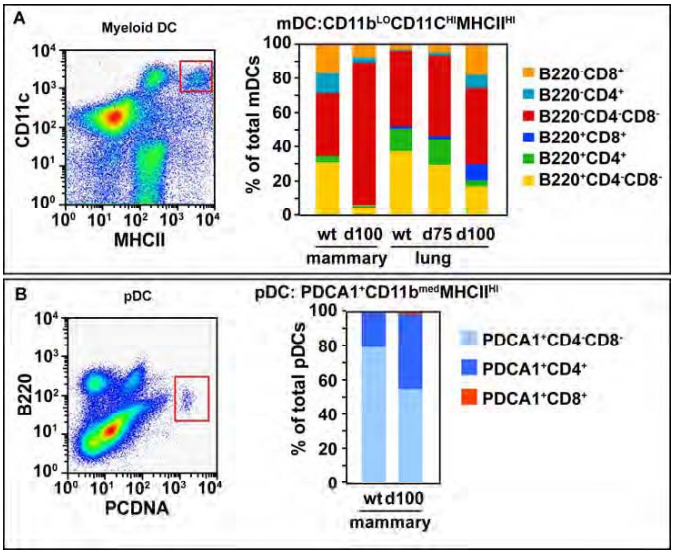


Figure 9: DC subpopulations in primary and metastatic mammary tumors. DC subsets in wildtype (wt) mammary and lung tissue, vs primary tumors from PyMT mice at d100, or d75 premetastatic vs. d100 metastatic lungs by FACS revealing A) $CD45^+CD11b^{Lo}CD11c^{Hi}MHCII^{Hi}$ mDCs, and B) $PDCA^+B220^+CD11b^{Mid}MHCII^{Hi}$ pDCs. N=3 mice/group.

Cytokine expression in $CD4^+$ T lymphocytes: Since our in vivo data implicated $CD4^+$ T cells as potentiators of metastasis, we sought to determine the cytokine profile of $CD4^+$ T cells in mammary glands and lungs of tumor naïve mice, as compared to those found in mammary tumors, lymph nodes and lungs tumor-bearing mice. Thus, we evaluated mRNA expression of $CD4^+$ T cells isolated from LNs and mammary carcinomas of 95 day-old *PyMT* mice (**Figure 10)** for transcription factors and effector molecules indicative of T_{reg}, T_H1, T_H2 or T_H17-type responses. $CD4^+$ T lymphocytes isolated from draining LNs (LNs) and mammary carcinomas of *PyMT* mice exhibited elevated expression of *GATA3* (T_H2) and *T-bet* (T_H1) mRNA, but not *FOXP3* (T_{reg}), when compared to LNs of wild type littermates indicating that both T_H1 and T_H2 effector lineages were expanded in LNs and in tumors. In order to assess the functional consequences of these, we assessed the cytokine expression profile of $CD4^+$ cells and found significant induction in T_H2 cytokines including *IL-4*, *IL-13,* and *IL-10* and to a lesser extent the T_H1 cytokine *IFNg* and by contrast, *IL-17a* was not significantly expressed (**Figure 10E-H**). These results were further confirmed by *ex vivo* activation of $CD4^+$ T lymphocytes (isolated from spleen, draining LNs and tumors of *PyMT* mice) with anti-CD3/CD28 Igs (data not shown). Analysis of expression of IL-4, IFNγ and IL-17 by ELISA, and IFNγ and IL-4 by intracellular flow cytometry, and found that activated $CD4^+$ T cells expressed higher levels of IL-4 as compared to IFNγ or IL-17 (**Figure 10 C and H**), and that IL-4-expressing $CD4^+$ T cells represented a larger fraction of the total $CD4^+$ T cells present in mammary tumors *in vivo* (data not shown). Moreover, we compared these expression characteristics with that found in

CD4+ T cells from lungs of tumor-naïve mice versus metastatic lungs of PyMT mice and found significantly distinct profiles (**Figure 10I-R)**. Our studies over the next funding cycle will seek to evaluate the functional significance of these differences.

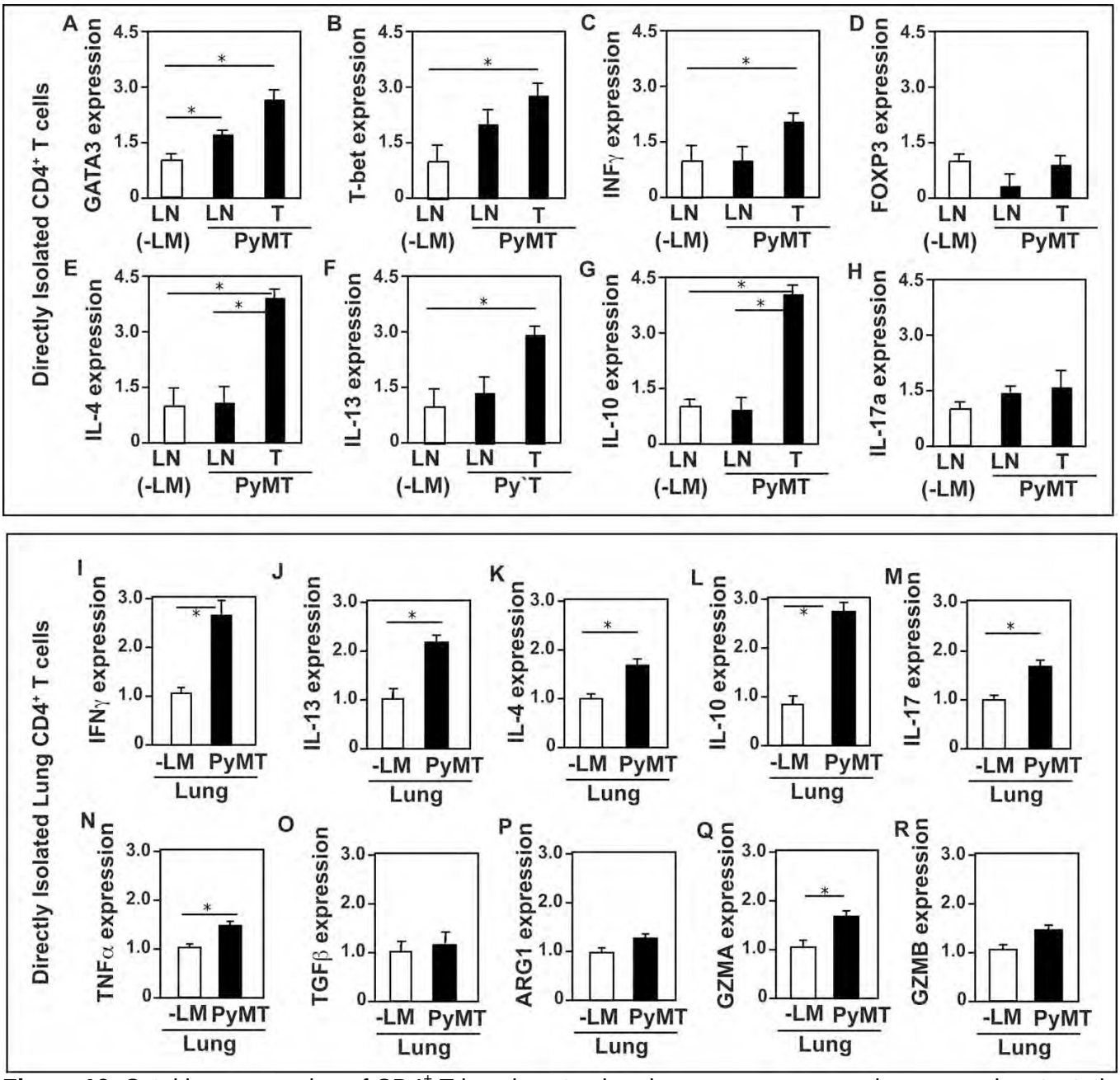


Figure 10. Cytokine expression of $CD4^+$ T lymphocytes in primary mammary carcinomas and metastatic lungs. A-H) Analysis of cytokine expression by tumor-associated $CD4^+$ T cells. $CD4^+$ T cells were isolated by flow sorting from lymph nodes and tumors of 95 day-old *PyMT* mice and corresponding negative littermates (n=4/cohort). Sorted cells were lysed and RNAs were assessed by qRT-PCR for *GATA3, T-bet, FOXP3, IFNγ, IL-4, IL-13, IL-10 and IL-17α* expression. Data is depicted as the mean fold change from the standardized sample (-LM lymph node). I-R) Analysis of cytokine expression in $CD4^+$ T cells isolated from lungs of tumor naïve (-LM) and tumor-bearing PyMT mice. $CD4^+$ T cells were isolated by flow sorting from represented tissues (n=5/cohort). Sorted cells were lysed and RNAs were assessed by qRT-PCR for *IFNγ, IL-4, IL-13, IL-10 and IL-17a, TNFα, TGFβ, GZMA, GZMB* expression. Data is depicted as the mean fold change from the standardized sample (-LM lung CD4s). SEM is shown and * denotes $p<0.05$ by t-test.

Transplantation model of breast cancer metastasis: Use of genetically engineered mouse models (GEMMs) of breast cancer metastasis are hampered by variable tumor latency, tumor penetrance, and significant primary tumor burden in all mammary glands. Thus, to evaluate efficacy of anti-cancer agents specifically aimed at minimizing metastasis, we adopted a transplantation strategy that circumvents these issues[19]. Terminal end buds, tumor fragments, or cell suspensions from PyMT or TAg mice are harvested, manipulated *ex vivo* to express Luciferase and/or fluorescent proteins, and transplanted into the mammary fat pad of naïve syngeneic mice, and tumor development monitored until palpable tumors appear and achieve a size of 1.5 cm (**Figure 11A**). Tumors are then surgically resected (**Figure 11B-C)** and metastasis evaluated at specific time points after removal of primary tumors (**Figure 11D-E**). In addition, utilization of Luciferase^{+}/PyMT or /TAg carcinoma cells allows for non-invasive monitoring of primary tumor burden and metastatic disease (**Figure 11F**). This model enables utilization of large cohorts of immune-competent mice bearing mammary tumors at the same stage of development and will facilitate our next series of studies where we neutralize TH2 immunity simultaneous with delivery of cytotoxic therapy, and attempt to induce a durable anti-tumor immune response. Delivery of therapeutic agents in a manner mimicking clinical therapy for women with breast cancer or high grade DCIS.

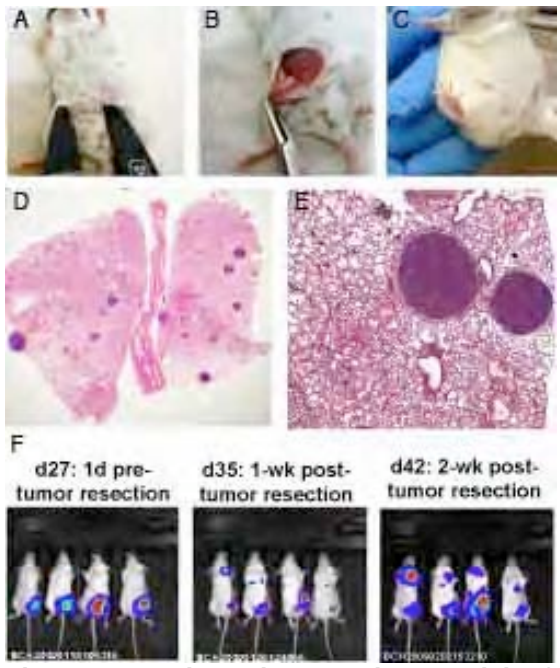


Figure 11: Orthotopic transplant model. A) d27 orthotopic tumor, **B**) mammary tissue post tumor resection, **C**) d35, 1-wk post tumor resection. **D**) Histology of d42 lungs from tumor-resected mouse showing metastases. **E**) High power mag. of metastatic tumor. **F**) Longitudinal imaging of luciferase expressing mammary tumor at d27, d35 and d42.

h. **Analyze role of proteases expressed by both tumor and stromal cells by crossing transgenic mice deficient in a protease already implicated in breast cancer progression with transgenic mice prone to development of mammary adenocarcinoma.**

i. Analyze role of proteases expressed by leukocytes by crossing transgenic mice deficient in a candidate leukocyte identified in Task 1 with transgenic mice prone to development of mammary adenocarcinoma. Months 1-24 (h-i)

To assess the functional significance of cathepsin C as a regulator of mammary carcinogenesis, we have generated breeding colonies of PymT mice harboring homozygous null mutations in the cathepsin C gene and

compared primary tumor development and pulmonary metastasis in age matched cohorts of PymT/cathepsin $C^{+/-}$ and PymT/cathepsin $C^{-/-}$ mice. Our data reveal that primary tumor latency and total primary tumor burden

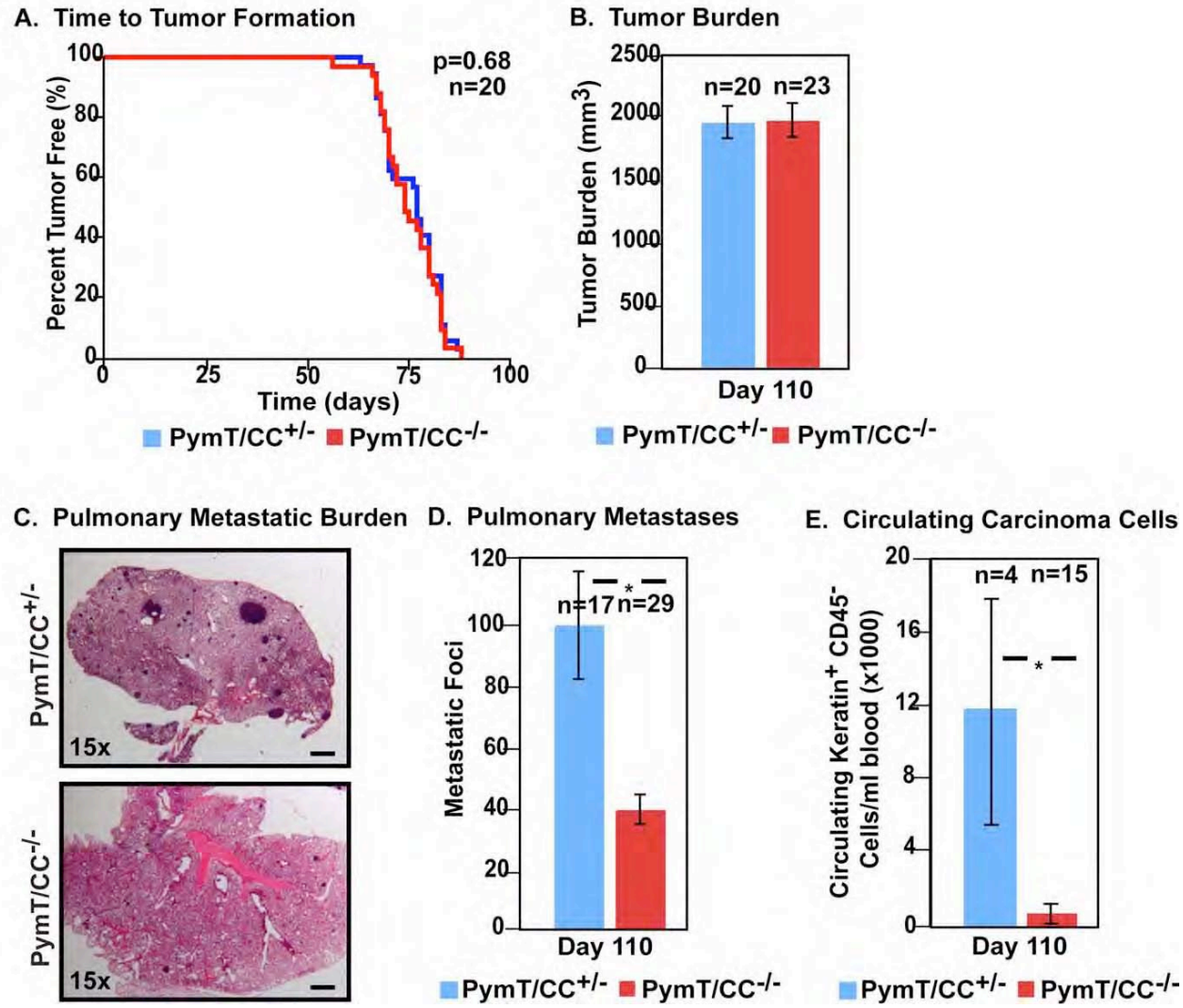


Figure 12: Cathepsin C regulates breast cancer metastasis. A) The percent of tumor free PymT/CC+/- (blue) and PymT/CC-/- (red) mice. B) Total mammary tumor burden in mice at day 110. C) Representative lung sections depicting metastatic tumor burden visualized by H&E staining. The scale bar represents 1.0 mm. D) Quantitation of average number of metastatic foci/5.0 mm^2 lung section. D) The number of circulating carcinoma cells accessed by flow cytometry using 200 ml of blood and staining for live, cytokeratin$^+$CD45$^-$ cells. Significance was determined by the Student's t test (*, $p<0.05$)

in PymT^{+}/cathepsin C$^{+/-}$ and PymT/cathepsin C$^{-/-}$ mice (>20 mice/experimental group) is unchanged (**Figure 12A,B)**. However, we have found that cathepsin C does significantly regulate pulmonary metastasis formation as shown in **Figure 12C-E.** We found that upon quantitatively assessing the average number of metastatic foci from 5.0 µm lung sections/mouse from 110 day old PymT^{+}/cathepsin C$^{+/-}$ and PymT/cathespin C$^{-/-}$ mice where each lung was completely sectioned and 6 sections evaluated every 100 µm apart by staining with H&E and total number of metastatic foci (greater then 5 cells) was quantified a statistically significant difference was found ($p<0.05$) by student t test (**Figure 12D)**. In addition, we revealed that the change in metastatic frequency was likely due to a change within the primary tumor microenvironment since the number of circulating carcinoma cells by FACS following blood draw by right heart puncture of terminal day 110 animals and evaluated live, cytokeratin positive, CD45 negative cells and found a statistical changes greater then $p<0.05$ as determined by student t test **(Figure 12E)**. While expression of cathepsin C does not regulate latency of primary tumor development, histopathological assessment of blood vessels in mammary tumors of cathepsin C-proficient versus –deficient/PymT mice reveals a significant difference in CD31-positive vessel numer within tumors and a change in overall vessel diameter (**Figure 13**). Over the next funding period, we will endeavor to reveal the functional significance of this observation as well as to reveal the specific aspects of tumor metastasis that cathepsin C regulates, as well as extending the results into human tissues to determine if cathepsin C represents a prognostic indicator of metastasis in women with breast cancer.

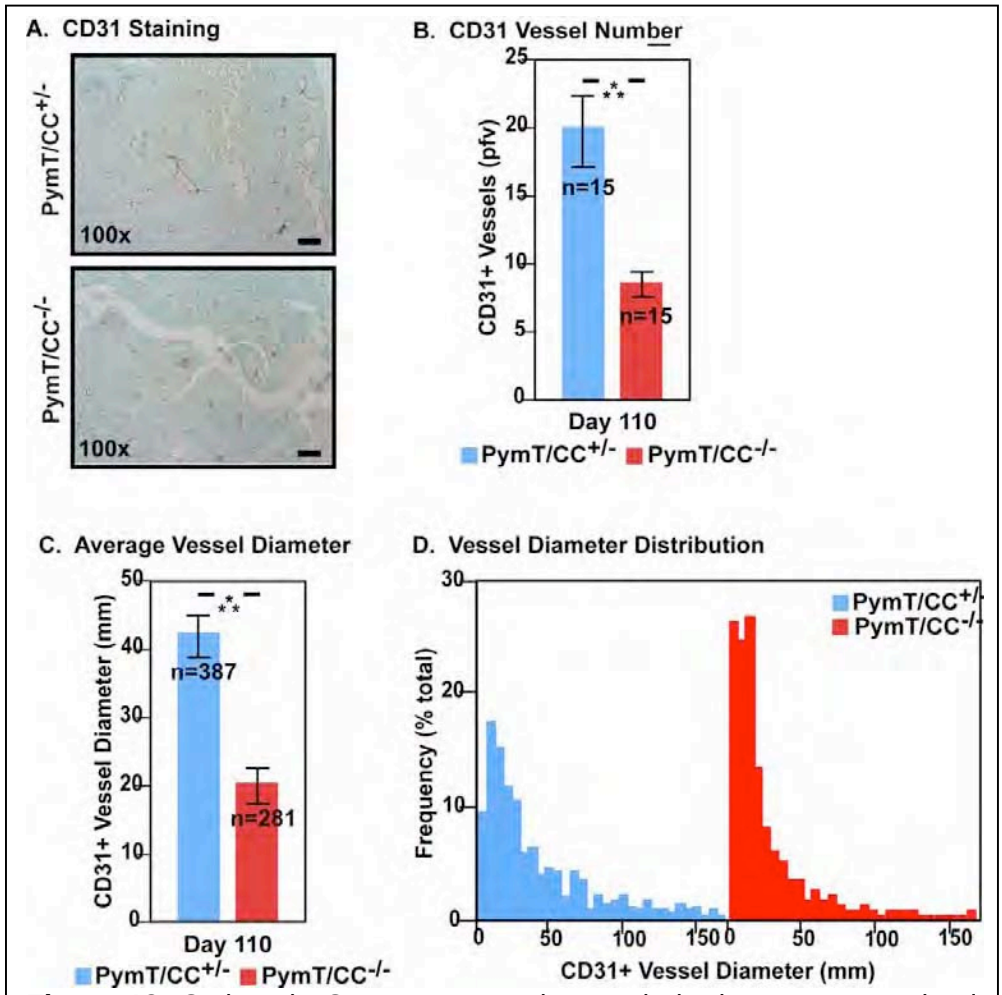


Figure 13: Cathepsin C promotes angiogenesis in the mammary gland during tumor development. A) Representative mammary gland sections depicting blood vessels visualized by CD31 staining. The scale bar represents 0.1mm. B) Quantitation of the average number of CD31 luminal structures/1.0 mm2 mammary gland section from PymT/CC+/- (blue) and PymT/CC-/- (red) mice. C) Average largest diameter of CD31 luminal structures from mammary glands D) Size distribution of CD31 luminal structures from mammary glands. CD31 structures were distributed into categories on the basis of their largest diameter and the frequency of these categories was determined based upon the total number of CD31 structures analyzed. Significance was determined by the Student's t test (*, $p<0.0005$)

Task 3. Develop non-invasive imaging reagents to monitor leukocyte and/or protease-specific events during mammary carcinogenesis

a. **Identify and characterize selective peptide substrates and selective binding peptides for proteases already implicated in breast cancer progression. (initial projection: Months 1-24; revised projection: Month 1-36**
b. **Identify and characterize selective peptide substrates and selective binding peptides for candidate proteases validated in Task 2. Months 24-48**

We are happy to report that the main focus of our work in this area has now been published[20]. The manuscript can also be found in Appendix B. Below reflects a brief summary of the work.

Matrix metalloproteinase-14 (MT1-MMP or MMP-14) is a membrane-associated protease implicated in a variety of tissue remodeling processes and a molecular hallmark of select metastatic cancers. The ability to detect MMP-14 in vivo would be useful in studying its role in pathologic processes and may potentially serve as a guide for the development of targeted molecular therapies. Four MMP-14 specific probes containing a positively charged cell penetrating peptide (CPP) d-arginine octamer (r8) linked with a MMP-14 peptide substrate and attenuating sequences with glutamate (8e, 4e) or glutamate- glycine (4eg and 4egg) repeating units were modeled using an AMBER force field method. The probe with 4egg attenuating sequence exhibited the highest CPP/attenuator interaction, predicting minimized cellular uptake until cleaved. The in vitro MMP-14 mediated cleavage studies using the human recombinant MMP-14 catalytic domain revealed an enhanced cleavage rate that directly correlated with the linearity of the embedded peptide substrate sequence. Successful cleavage and uptake of a technetium-99m labeled version of the optimal probe was demonstrated in MMP-14 transfected human breast cancer cells. Two-fold reduction of cellular uptake was found in the presence of a broad spectrum MMP inhibitor. The combination of computational chemistry, parallel synthesis and biochemical screening, therefore, shows promise as a set of tools for developing new radiolabeled probes that are sensitive to protease activity.

As indicated in our 2008 Progress Report, the departure from UCSF of our collaborator Dr. Ben Franc has made it difficult to precede further in this area; thus, we have re-grouped, and are pursuing an alternative approach to visualize “inflammation” non-invasively with Dr. Heike Daldrup-Link, a Radiologist at UCSF. Our new approach will take advantage of Dr Daldrup-Link’s tremendous expertise with cell-based labeling for non-invasive imaging. As such, details of our ongoing work is described below.

c. **Synthesize novel fluorescent probes for imaging protease activity using peptide substrates identified above (3.a.). Months 6-48**

Based upon our compelling data implicating cathepsin C as an important regulator of late-stage mammary carcinogenesis, we generated with our collaborator Matt Bogyo (Stanford University)[16] a novel highly selective activity-based probe to assess relative changes in cathepsin C activity in tissue lysates isolated from distinct stages of neoplastic progression. We are currently utilizing this probe to assess cathepsin C activity in tissue lysates from distinct stages of progression in MMTV-PymT mice, and we are in the process of re-synthesizing a new fluorescent probe, as was done previously by the Bogyo group for evaluating cathepsin B in pancreatic islet tissue[21], to evaluate relative cathepsin C activity in tissue ex vivo. For the reasons mentioned above regarding our focus over the previous 12 months on investigating leukocytes during mammary carcinogenesis, this aspect of the project was not pursued vigorously over the previous 12 month period. We anticipate its completion by month 48 as initially projected.

d. **Demonstrate ability of fluorescently-labeled molecules to localize to xenograft and/or 3-dimensional organotypic cancer models using confocal fluorescence and/or whole-body fluorescence imaging. Months 6-36**

To initiate these studies, and based on our compelling preliminary data demonstrating functional roles for monocytes in regulating late-stage mammary cancer metastasis, in collaboration with Dr Daldrip-Link, we have investigated whether fluorescently-labeled monocytes could be used for optical imaging of peri-tumoral

inflammation since non-invasive evaluation may have diagnostic value and assist with therapeutic monitoring. Thus, 5- to 10 million murine monocytes were labeled with DiD (a fluorescent carbocyanine dye) and injected intravenously into MMTV-PymT tumor-bearing mice and age-matched negative littermate control mice. Optical imaging was performed before, directly after, and at 1, 2, 6 and 24 hours after cell injection. Uptake in body organs was evaluated to determine technical success. Ratios of post-injection to pre-injection fluorescent signal intensity (SI post/pre) of the tumors (MMTV-PymT mice) and mammary tissue (/n controls) were calculated. Confocal fluorescent microscopy was used to confirm that labeled cells were present within tumors after intravenous injection.

Technical success was obtained in all 12 mice, with increasing fluorescence seen in liver, spleen and lungs of all animals after labeled-monocyte injection. MMTV-PymT mice showed progressing tumor fluorescence up to 24 hours after cell injection, with average SI post/pre ratios of 1.8+/- 0.2 (range, 1.1-2.6). Control mice showed no increased fluorescence in mammary tissue after monocyte injection with average SI post/pre ratios of 1 +/- 0.0 (range, 1.0 to 1.0) (**Figure 14**). The difference between averages was found to be statistically significant, with a p-value of 0.01. Confocal fluorescence microscopy confirmed the presence of intravenously injected DiD-labeled cells within the breast tumors (**Figure 14D**). From these initial studies, we conclude that i.v. delivery of congenic monocytes will accumulate at sites of mammary cancer development in PymT mice, providing proof of principle that peri-tumoral inflammation can be evaluated using optical imaging. Further studies will be needed to clarify if this novel imaging parameter will provide prognostic or therapeutic information and to what degree we can target specific subpopulations of leukocytes in primary tumors as opposed to pulmonary metastases.

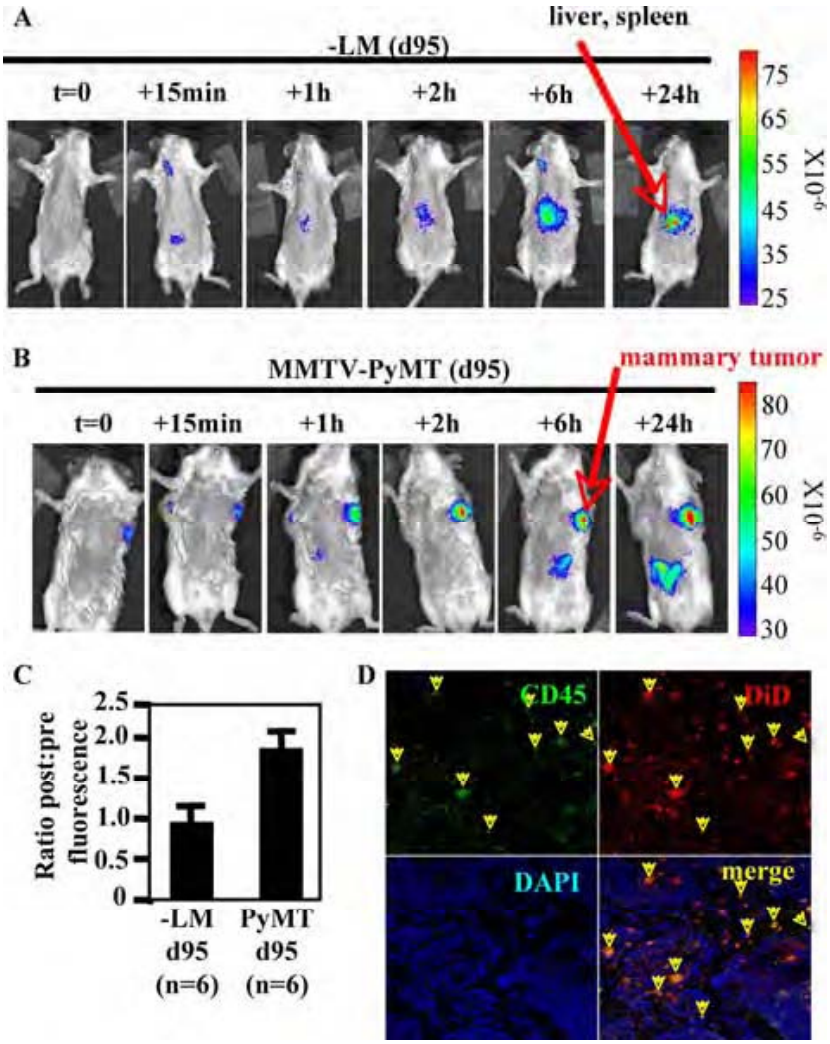


Figure 14: Homing of DiD-labeled monocyts to mammary tumors. OI of d95 wt (A) and PyMT (B) mice showing liver and mammary homing of DiD-labeled monocytes, revealing a 2-fold enhancement in fluorensence (C). Immunofluorescent-detection of DiD-labeled monocytes (red) in mammary tissue sections confirms their identity as CD45+ leukocytes (D).

Breast cancer development is accompanied by increased infiltration of mammary tissue by macrophages[22], or so called tumor-associated macrophages (TAMs). TAMs are significant for tumor progression as macrophage-deletion studies have revealed slowed tumor development and attenuated pulmonary metastasis in their absence[8]. In addition, we have recently shown that macrophages differentially respond to T_H1 versus T_H2-type cytokines, and accordingly, T_H2-activated macrophages exhibit tumor-promoting bioactivities, whereas T_H1-activated TAMs, interfere with tumor progression and significantly attenuate pulmonary metastasis formation[22]. In addition, DePalma and colleagues recently reported that monocytes engineered to express interferon (IFN)-α, home to mammary tumors where they exhibit significant anti-tumor activity resulting in attenuated tumor growth and metastasis[23]. Based on these studies, we will

investigate if transient Alk-5 blockade (see below) can be exploited to increase tumor infiltration of autologous peripheral blood (PB)-isolated monocyte/macrophages, and if so, can that capability be exploited to deliver an anti-cancer molecule to tumor tissue and thereby limit and/or block tumor growth. Clinical trials using injection of autologous macrophages as cell-based therapy into tumor patients have previously been validated as safe with limited adverse side effects[24].

e. **Identify and characterize covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies that uniquely interact with target immune cell populations. Months 12-48.**

Tumor accumulation of intravenously administered macromolecular compounds, imaging agents and some cytotoxic drugs, depends on multiple variables including blood flow, transendothelial solute/drug permeability, interstitial fluid pressure (IFP), and interstitial volume. Since the altered permeability of tumor microvessels limits delivery of macromolecular cytotoxic drugs, and high IFP in solid tumors further reduces drug extravasation from vessels to tumor interstitium[25], a major clinical goal is development of novel delivery strategies that overcome tissue perfusion/accumulation barriers to enhance drug delivery and improve clinical outcome. The development of appropriate strategies to overcome this challenge is further complicated by paradoxical studies revealing that in some contexts restricting, versus enhancing tumor vessel permeability may lead to increased tissue perfusion and enhanced accumulation/delivery of macromolecular imaging agents or cytotoxic drugs in solid tumors. For example, transient increases in tumor perfusion and improved penetration of chemotherapeutic agents has been achieved by 'normalization' of tumor vasculature using antagonists of vascular endothelial growth factor (VEGF) and/or its receptors (VEGFR1 and 2)[26]. Whereas sustained anti-VEGF treatment can inhibit angiogenesis and reduce vascular density, short-term blockade of VEGF can transiently reduce permeability, stabilize tumor vessels via recruitment of pericytes, and improve hemodynamics. However, these effects are largely limited to early stage tumors containing immature vessels lacking pericytes[27].

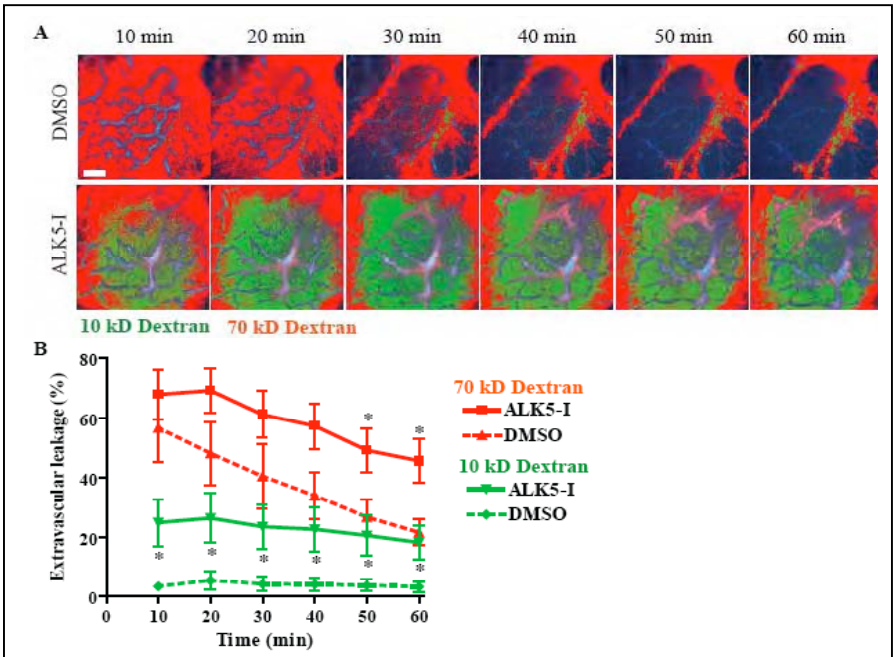


Figure 15: Alk5-blockade enhances intratumoral delivery of high MW dextran. A) Leakage of high (70kD, green) and low (10kD, red) MW dextrans in tumors of d95 PyMT mice treated or untreated with Alk5-inhibitor. Blue reflects non-targeted quantum dots to differentiate intra- vs extravascular space. B) Quantitative assessment of dextrans in PyMT tumors at indicated times after i.v. injection (n=5/group). * $p<0.05$.

Paradoxically, agents that directly promote vascular leakage and increase tumor microvascular permeability, can potentiate delivery and/or accumulation of cytotoxic therapies in tumor interstitium by overcoming high IFP and allowing convection driven uptake of large macromolecular agents into tissues[28]. To this end, we have discovered a novel endogenous pathway regulating vascular leakage that remains functional in both early and later stage breast cancers; thus, we predict that this pathway can be exploited to improve tissue perfusion and tumoral drug delivery of high molecular weight (MW) macromolecular compounds. Using transgenic models of de novo carcinogenesis (K14-HPV16 and MMTV-PyMT), we found that inhibiting vascular transforming growth factor beta 1 (TGFβ_1) or its type I receptor, Alk5, leads to increased tumor vessel permeability in both early and late stage carcinomas (**Figure 15**)[29]. Moreover, Alk5 blockade primarily increases permeability in more mature larger diameter vessels that have substantial mural cell coverage. Although Alk5 blockade does not modify

pericyte coverage, it regulates the frequency of endothelial cell gaps that permit passage of macromolecules. Therefore, we hypothesize that blockade of Alk5 in tumor vessels will improve tissue perfusion and penetration of conventional high MW chemotherapeutic agents, and that this approach will be applicable to both early and late stage breast tumors and *improve overall patient survival.* Thus, over the next funding cycle, we will investigate if transient Alk5-blockade in MMTV-PyMT mice, or mice bearing human breast cancer xenograph tumors, improves delivery of labeled-monocytes and/or high molecular weight imaging compounds (see below) to tumor interstitium.

f. Validate covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies for selectivity in the organotypic 3-dimensional co-culture models. Months 12-48.

A major challenge for improving cytotoxic drug delivery to tumors is to identify patients that would most likely benefit from adjuvant therapies to alter vascular permeability and tissue perfusion, and to prospectively predict the subsequent therapeutic outcome. Since tumor-specific and even regional intratumoral variations in microvessel characteristics may prove pivotal for tumor response to therapy, new delivery strategies must take regional parameters into account. Currently, a complete pathological response (cPR) of treated primary tumors is the most significant therapeutic endpoint and strongly correlates with patient survival[30]. However, this indicator can only be assessed after completion of neoadjuvant chemotherapy and surgery. Clinicians and researchers have long sought a surrogate to accurately predict outcome to therapy thus enabling personalized tailoring of cytotoxic drug regimens. Magnetic resonance (MR) imaging is a highly accurate technique for predicting cPR and a recent study comparing palpation, mammography, ultrasound and MR as predictors of cPR showed 19%, 26%, 35% and 71% agreement, respectively, with cPR[31]. Moreover, MR imaging offers the potential to noninvasively and serially monitor characteristics of tumor microvessels, including apparent transendothelial permeability-surface area products (K^{ps}) and richness of vascularity (fPV), parameters that are important when considering tumoral delivery and accumulation of cytotoxic drugs (macromolecular or macromolecular-complexed) to tumor interstitium. Macromolecular contrast medias (MMCM), such as albumin-Gd-DTPA with a MW of 60-90 Kilodaltons, and iron oxide nanoparticles with a size of 30-150 nm, can detect, quantify and grade microvessel hyperpermeability of tumors to macromolecules. In contrast to standard small molecular contrast agents for MR imaging, MMCMs are able to predict tumor therapy responses to vascular targeting drugs as early as one-day after treatment based on diagnosed changes in microvascular permeabilities[32]. Kinetic analysis of dynamic MMCM-enhanced MR data in breast tumor patients has been shown to provide useful estimates of the angiogenesis status of cancers including tumor blood volume and microvascular permeability, to differentiate benign from malignant lesions, and to predict, as well as monitor tumor response to therapy[28,33]. Importantly, dynamic MR imaging can be utilized to assess regional and intratumoral variations in microvessels, and thus can be used to predict the accumulation and/or response to agents influencing vascular leakage given either as single agents, or given in combination with standard chemotherapies. Therefore in the next funding cycle, we will evaluate quantitative estimates of tumor microvascular permeability as determined by MMCM-enhanced MR imaging as a surrogate to predict tumor accumulation and/or response of macromolecular cytotoxic drugs following Alk5 blockade in early and late mammary carcinomas. In addition, we will directly monitor response to therapy via histopathologic analyses, latency of progression, and overall survival to demonstrate not only that inhibition of Alk5 improves cytotoxic drug accumulation, but also to demonstrate that the MR-based predictions correlate with the therapeutic outcome**. Thus, by exploiting a novel endogenous pathway we have revealed that regulates vascular permeability and which remains functional during breast tumor progression, we anticipate significantly improving delivery of both chemotherapeutic agents, as well as molecular imaging agents that will improve diagnosis and monitoring of early and late stage breast cancers, and thereby improve patient survival**.

Expression of folate receptors (FR) on breast cancer cells has been identified as a strong predictor of poor outcome in patients with breast cancer[34]. After adjustment for tumor size, nodal and estrogen receptor (ER) status, histology and tumor grade, strong staining for the FR remained significantly associated with poor outcome[34]. Folate is a key factor for DNA replication and cell proliferation. Breast cancer cells use the FR as their major and distinct route for folate entry into the cell. Conversely, non-neoplastic cells use a different route, the reduced folate carrier as their major transmembrane transporter of reduced forms of folate[35]. Thus,

because of its relative tumor-specificity and potential as a new prognostic marker, the FR has evolved into a major target for tumor imaging, and a new targeted cancer therapeutic.

Initial approaches for imaging FR on cancer cells have been performed with FR-targeted radiotracers, which provided a high sensitivity for detection of human nasopharyngeal, breast and ovarian carcinoma, but with limited spatial resolution and considerable radiation exposure[36]. FR-targeted optical imaging probes provided a similar sensitivity without radiation exposure, but were associated with limited anatomical resolution as well as not yet being clinically applicable[37]. More recently, FR-targeted contrast agents have been developed for magnetic resonance (MR) imaging[38,39]. MR is a clinically applicable technique with high anatomical resolution and no radiation exposure[38,40]. However, initial development of FR-targeted gadolinium chelates showed a limited MR signal effect[38,39,41]. One significant reason for this limited signal effect was the limited transendothelial permeability of these high molecular weight compounds into the tumor interstitium.

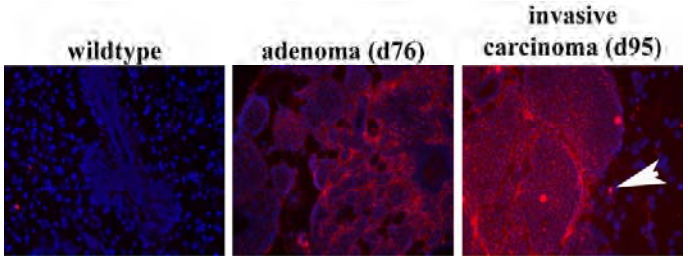


Figure 16: Increased FR expression in PyMT carcinomas. Immunodetection of FR (red staining) in mammary tissue of wildtype mice (left), and PyMT mice with an early stage (d76) adenoma (middle) versus late-stage invasive carcinoma (right). Blue staining (DAPI) reveals nucleated cells. Arrow: peritumor macrophage. Mag.all images: 40X

We have recently revealed that as mammary adenocarcinomas develop in MMTV-PyMT mice, expression of FR increases in tumors and is predominantly localized in malignant epithelial cells and peri-tumor immune cells, mostly macrophages (**Figure 16**). We will utilize FR-selective uptake of a novel FR-targeted MR contrast agent *P1133,* and its accumulation and retention in FR-positive versus FR-negative human breast cancer xenograph tumors to visualize tumor burder, as well as evaluate macrophage infiltration. *P1133* is an ultra small superparamagnetic iron oxide (USPIO) contrast agent, developed by Guerbet Research (France) (**Figure 17**), that Dr Daldrup-Link has previously used to detect FR-positive breast cancer xenograph tumors in athymic Harlan rats (Reinhard and Daldrup-Link, manuscript in preparation). *P1133* consists of an iron-oxide core and a hydrophilic coat conjugated to folate moieties. As a control, we will use *P904* that represents a non-FR-targeted analog of *P1133*, composed of an identical iron oxide core without the folate moiety, and has the same molecular properties as *P1133*. Thus, we will first conduct pilot studies to optimize MR imaging and kinetic analysis of both compounds in immune-deficient mice bearing MDA-MB-231 human beast cancer xenograph tumors to determine optimal MRI scanning parameters as indicated below.

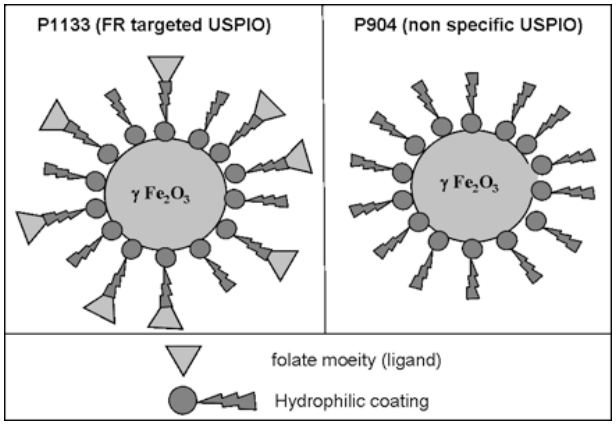


Figure 17: FR-targeted MR contrast agents. *P1133* consists of an iron-oxide core conjugated to a folate moiety, lacking in *P904*. Both agents have a hydrophilic coating.

g. **Validated covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies *in vivo* in the parental, immune and/or protease-modified mouse models of mammary carcinogenesis. Confirm relationship between level of nanoparticle uptake within tissues and level of immune cell infiltration histologically. Months 12-48.**

These studies will be initiated in the next funding cycle based on preliminary pilot studies as described above in sections d-f.

h. **Analyze evolution of leukocyte infiltration and/or protease expression in mouse mammary models using fluorescently-labeled molecular probes where animals are imaged longitudinally on weekly intervals. Months 12-60.**

These studies will be initiated in the next funding cycle based on preliminary pilot studies as described above in sections d-f.

i. **Select candidate molecular probes emerging from above (3.d.) that demonstrate the capability of being detected *in vivo* and are present at key timepoints in the evolution of breast cancer and use as a platform for development of protease-specific radiolabeled probes for single photon emission computed tomography (SPECT). Months 12-60.**

j. **Demonstrate ability of agents in 3.i. to localize to tumors in proportion to the level of the specific protease targeted using *in-vivo* SPECT-CT imaging (X-SPECT, Xenogen Corp.) with *ex-vivo* autoradiography, scintillation well-counting, and immunohistochemistry to pathologically confirm levels of tissue expression and/or immune cell infiltration in areas concentrating the radiolabeled probe. Months 24-60.**

k. **Monitor relationships between presence of various immune cell populations and protease expression within mouse mammary models utilizing multi-modality imaging (MRI, SPECT-CT) in combination with the immune cell (MR) agents and protease (SPECT-CT) agents developed in 3.e. and 3.i., respectively. Months 36-60.**

These studies (i-k) have not yet been initiated

III. KEY RESEARCH ACCOMPLISHMENTS:

Task 1. Define the profile and proteolytic contribution of leukocytes in human breast cancer and in transgenic mouse models of mammary carcinogenesis.

a. Elucidate the spectrum of CD45$^+$ cells in normal and neoplastic human breast tissues.

Months 1-12

- Acquired representative paraffin-embedded samples of human mammary tissue reflecting disease-free, ductal carcinoma in situ, and frank carcinoma (10 each)
- Performed histochemical evaluation by hematoxylin and eosin staining to affirm tissue and disease stage
- Performed Immunodetection analysis on tissue sections to reveal leukocyte infiltrates, e.g., CD45, CD4, CD8, CD68, neutrophil elastase, mast cell chymase, and CD31 for endothelial cells

Months 12-24:

- Initiated collaboration with Drs Susan Love and Lisa Bailey to acquire freshly resected breast cancer tissue and adjacent normal control tissue to utilize for FACS analysis

Months 24-36:

- Evaluated a tissue microarray containing 179 specimens of invasive breast cancer to evaluate if CD4, CD8 and CD68-positive leukocyte infiltrations correlated with overall 5-year patient survival
- Currently evaluating a tissue microarray containing 498 specimens of invasive breast cancer to evaluate if CD4, CD8 and CD68-positive leukocyte infiltrations correlated with overall 5-year patient survival

b. Elucidate the spectrum of CD45$^+$ cells in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.

Months 1-24

- Generated breeding colony of MMTV-PymT mice and aged out to representative endpoints to collect tissues reflecting early mammary intraepithelial neoplasia (MIN), DCIS and carcinoma
- Performed histochemical evaluation by hematoxylin and eosin staining to affirm tissue and disease stage
- Performed Immunodetection analysis on tissue sections to reveal leukocyte infiltrates, e.g., CD45, CD4, CD8, CD68 and F4/80 for macrophages, 7/4 and CD11b for myeloid cells, CD119 for mast cells, and CD31 for endothelial cells

- Performed flow cytometric analyses on staged tissue suspensions from MMTV-PymT mice to quantitatively evaluate immune cell infiltrates at each stage of neoplastic progression.

Months 12-24

- Extended FACS analysis to include examination of leukocytes populations in blood and spleen in tumor bearing mice MMTV-PymT mice
- Performed histochemical evaluation by hematoxylin and eosin staining to affirm tissue and disease stage in PymT/RAG1$^{-/-}$, PymT/JH$^{-/-}$, PymT/CD4$^{-/-}$/CD8$^{-/-}$, PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$ mice
- Performed Immunodetection analysis on tissue sections to reveal leukocyte infiltrates, e.g., CD45, CD4, CD8, CD68 and F4/80 for macrophages, 7/4 and CD11b for myeloid cells, CD119 for mast cells, and CD31 for endothelial cells in PymT/RAG1$^{-/-}$, PymT/JH$^{-/-}$, PymT/CD4$^{-/-}$/CD8$^{-/-}$, PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$ mice
- Performed flow cytometric analyses on staged tissue suspensions from PymT/RAG1$^{-/-}$, PymT/JH$^{-/-}$, PymT/CD4$^{-/-}$/CD8$^{-/-}$, PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$ mice to quantitatively evaluate immune cell infiltrates at each stage of neoplastic progression

Months 24-36:

- Published DeNardo et al., Cancer Cell 2009 reporting on the functional significance of CD4+ T cells and macrophages as mediators of breast cancer metastasis

c. Develop a profile of proteolytic activities in normal and neoplastic human breast tissues.

Months 1-12

- Collaborated with the Sloane BCCOE to evaluate mRNA isolated human mammary tissue reflecting disease-free tissue, DCIS and Stage I, II and II disease on the Hu.Mu protease chip array

d. Develop a profile of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.

Months 1-24

- Collaborated with Prof. Dylan Edwards to evaluate mRNA expression for cysteine cathepsin gene family members on staged tissues from MMTV-PymT mice

e. Determine cellular origins of proteolytic activities in normal and neoplastic human breast tissues.

Months 24-36

- Performed Immunodetection analysis on tissue sections to reveal cathepsin C expression characteristics during mammary carcinogenesis in human invasive breast cacner

f. Determine cellular origins of proteolytic activities in normal and neoplastic mammary tissues from mouse models of mammary carcinogenesis.

Months 1-36

- Evaluated origin of cathepsin C expression in MMTV-PymT tissue sections
- Used double Immunoflorescence staining to localize cathepsin C expression to infiltrating F4/80+ myeloid cells in metastatic lungs of PymT mice

g. Compare immune cell infiltrations in mouse and human normal and neoplastic tissues. Identify candidate cell types for further study.

Months 1-24

- Published in DeNardo et al., Cancer Cell 2009 identifying CD4+ T cells and macrophages as functionally significant

Months 24-36:

- Based on DeNardo et al 2009, Eevaluated a tissue microarray containing 198 specimens of invasive breast cancer to evaluate if CD4, CD8 and CD68-positive leukocyte infiltrations correlated with overall 5-year patient survival

h. Compare proteolytic activities in mouse and human normal and neoplastic tissues based. Identify candidate protease activities for further study.

Months 1-24

- Studies examining comparative expression of cathepsin C initiated

Task 2. Validate target molecules and/or specific immune cell types in biological assays and in animal models of mammary carcinogenesis.

a. Establish 3-dimensional mammary epithelial organotypic cell culture model system cultured on basement membrane.

Months 1-9

- Dr. DeNardo was trained in development, isolation, culture and maintenance of 3D organotypic cultures by Dr. Jay Debnath (UCSF).
- Routine generation, culture and maintenance of 3D organotypic cultures established in Coussens.
- Routine isolation of primary MECs from wt and MMTV-PymT mice established in Coussens lab and subsequent culture in 3D routine.

b. Establish routine isolation and short-term culture conditions for mouse leukocytes.

Months 1-9

- Routine isolation and culture of primary macrophages, T cells, immature myeloid cells, macrophages and mast cells established in Coussens lab from peripheral blood, spleen, wt mammary glands and early and late-stage carcinomas from MMTV-PymT mice.

c. Establish 3-dimensional organotypic co-culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic cell types.

Months 6-12

- 3D co-culture of nMECs and pMECs with naïve and tumor-associated macrophages established in Coussens lab
- 3D co-culture of nMECs and pMECs with naïve and tumor-associated macrophages and CD4+ T cells established in Coussens lab

d. Establish 3-dimensional organotypic culture model system for analysis of paracrine interactions between mammary epithelial cells and specific leukocytic-derived proteases.

Months 6-12

- Paracrine regulation of MECs by T cells and macrophages identified as being metalloproteinase-dependent

e. Analyze role of leukocytes in regulating mammary epithelial morphogenesis, proliferation, cell death, apicobasal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in normal and oncogene-expressing 3-dimensional acini cultured on basement membrane.

Months 12-24

- Acinus disruption assay validated in Coussens lab
- Paracrine regulation of MECs by CD4+ T cells and macrophages identified as IL4-dependent
- Paracrine regulation of MECs by CD4+ T cells and macrophages identified as utilizing Shh and Wnt proteins
- Paracrine regulation of MECs by CD4+ T cells and macrophages is due to differential activation of T_H2-type cytokines versus repression of T_H1-type cytokines
- Paracrine regulation of MECs by CD4+ T cells and macrophages involves increased expression of EGF

Months 24-36:

- Published DeNardo et al., Cancer Cell 2009 reporting on the functional significance of CD4+ T cells and macrophages as mediators of breast epithelial cell invasion into matrix using 3D assay
- Examined small molecular weight cFMS and CSF1R inhibitors in pilot studies to examine efficacy in regulating macrophage-epithelial cell interactions

f. Analyze role of leukocyte-derived proteases in regulating mammary epithelial morphogenesis, proliferation, cell death, apical-basal polarity, cell-cell adhesion, and formation of invasive and protrusive structures in

normal and oncogene-expressing 3-dimensional acini cultured on basement membrane.

Months 12-36

- Initiated in vivo assessment of cathepsin C as mediator of breast carcinogenesis
- Revealed that MMTV-PyMT mice lacking cathepsin C have diminished presence of angiogenic blood vessels in primary tumors
- Revealed that MMTV-PyMT mice lacking cathepsin C have diminished presence of malignant circulating carcinoma cells in peripheral blood and diminished growth of pulmonary metastases

g. Analyze role of candidate leukocytes by crossing mice deficient in or modified such that individual or classes of leukocytes (identified in Task 1) are deficient with transgenic mice prone to development of mammary adenocarcinoma.

Months 1-12

- MMTV-PymT mice intercrossed with RAG1$^{-/-}$ mice
- MMTV-PymT mice intercrossed with CD4$^{-/-}$CD8$^{-/-}$ mice
- MMTV-PymT mice intercrossed with JH$^{-/-}$ mice
- Evaluate primary tumor and pulmonary metastasis development in crosses
- T cells identified as significant adaptive leukocyte regulating pulmonary metastasis formation

Months 12-24

- MMTV-PymT mice intercrossed with CD4$^{-/-}$ mice
- MMTV-PymT mice intercrossed with CD8$^{-/-}$ mice
- Assessment of neoplastic progression in PymT/RAG1$^{-/-}$, PymT/JH$^{-/-}$, PymT/CD4$^{-/-}$/CD8$^{-/-}$, PymT/CD4$^{-/-}$, PymT/CD8$^{-/-}$ mice
- Metastasis in PymT mice found to be CD4+ T cell-dependent
- CD4+ T cells found to repress TH1-type cytokine expression by tumor-associated macrophages
- CD4+ T cells found to induce TH2-type cytokine expression by tumor-associated macrophages

Months 12-36

- Revealed that CD4+ T cells potentiate breast cancer metastasis by expression of IL-4
- Revealed that treating MMTV-PyMT mice with neutralizing antibodies to IL_4 significantly attenuates growth of pulmonary metastases
- Revealed that activation of the IL-4Rα expressed on macrophages induces high level expression of EGF
- Revealed that macrophage-EGF expression is required for breast cancer metastasis in MMTV-PyMT mice
- Pilot studies examining efficacy of cFMS and CSF1R inhibitors to block metastasis on MMTV-PyMT mice
- Established organotypic breast carcinoma pulmonary metastasis model

h. Analyze role of proteases expressed by both tumor and stromal cells by crossing transgenic mice deficient in a protease already implicated in breast cancer progression with transgenic mice prone to development of mammary adenocarcinoma

Months 1-12

- MMTV-PymT mice intercrossed with cathepsin C$^{-/-}$ mice

Months 12-24

- Generate cohorts of MMTV-PymT/cathepsin C$^{-/-}$ and +/- control mice to evaluate functional significance of cathepsin C during mammary carcinogenesis

Months 24-36

- Histopathological, immunological and biochemical characterization of cohorts of MMTV-PymT/cathepsin C$^{-/-}$ and +/- control mice to evaluate functional significance of cathepsin C during mammary carcinogenesis
- FACS analysis comparing infiltrating leukocyte populations in PyMT/cathepsin C-proficient vs –fedicient mice

i. Analyze role of proteases expressed by leukocytes by crossing transgenic mice deficient in a candidate

leukocyte identified in Task 1 with transgenic mice prone to development of mammary adenocarcinoma.

Months 12-24

- Breeding colonies of PymT/cathepsin C +/- and -/- mice generated, expanded and aged out to endpoints
- PymT/cathepsin C -/- mice found to have significantly reduced incidence of pulmonary metastasis
- Cohorts of PymT/cathepsin C +/- are still aging out for completion
- Cathepsin C found to be a significant regulator of pulmonary metastasis formation

Months 24-36

- Detailed analysis of leukocytes infiltrating cohorts of MMTV-PymT/cathepsin $C^{-/-}$ and +/- control mice to evaluate functional significance of cathepsin C in individual leukocyte types

Task 3. Develop non-invasive imaging reagents to monitor leukocyte and/or protease-specific events during mammary carcinogenesis

a. *Identify and characterize selective peptide substrates and selective binding peptides for proteases already implicated in breast cancer progression.*

Months 1-24

- RDM designed using MMP14-cleavage peptides
- RDM biochemically assessed for selective MMP-14 cleavage

Months 24-36

- Publish Watkins et al., 2009
- Pilot studies utilizing non-invasive Luciferase imaging to monitor breast cancer metastasis in organotypic model

b. *Identify and characterize selective peptide substrates and selective binding peptides for candidate proteases validated in Task 2.*

Months 24-48

- Initiate re-synthesis of FY01 cathepsin C activity probe with fluorescent tag for ex vivo and in vivo analyses

Months 24-36

- Validate folate receptor expression on breast carcinoma cells and infiltrating macrophages in MMTV-PyMT mice
- Validate transient Alk5-blockade as efficacious tool to enhance delivery of high molecular weight compounds to tumor interstitium

c. *Synthesize novel fluorescent probes for imaging protease activity using peptide substrates identified above (3.a.).*

d. *Demonstrate ability of fluorescently-labeled molecules to localize to xenograft and/or 3-dimensional organotypic cancer models using confocal fluorescence and/or whole-body fluorescence imaging.*

Months 6-12

- RDM optically screened in cell-based cultures
- Selective uptake of RDM affirmed in cell-based analyses
- Bodipy-RDM probe evaluated in murine breast cancer xenograft and selectivity analyses initiated

Months 12-24

- Monocytes labeled with DiD
- In vitro optical imaging validated of monocytes validated
- FACS analysis of labeled monocytes to confirm cell surface marker expression

Months 24-36

- Pilot experiments to evaluate efficacy of labeled monocytes as cell-based delivery vehicles carrying anti-cancer agents

e. *Identify and characterize covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies that uniquely interact with target immune cell populations.*

Months 1-12
- Not initiated in months 1-12

Months 12-24
- Not initiated in months 12-24

Months 24-36
- Not initiated in months 24-36

f. Validate covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies for selectivity in the organotypic 3-dimensional co-culture models.

Months 12-48
- Not initiated in months 1-36

g. Validated covalently coated iron oxide nanoparticles with poly(ethylene glycol) polymers functionalized with specific peptides or antibodies in vivo in the parental, immune and/or protease-modified mouse models of mammary carcinogenesis. Confirm relationship between level of nanoparticle uptake within tissues and level of immune cell infiltration histologically.

Months 1-12
- Not initiated in months 1-12

Months 12-24
- Optical imaging of DiD-labeled monocytes in MMTV-PymT and control mice
- Confocal microscopy of tissue sections to confirm presence of DiD-labeled monocytes in mammary tumors
- Quantitative analysis of fluorescence from mammary tumors following injection of DiD-labeled monocytes

Months 12-24
- Validate that transient Alk5-blockade enhances delivery of high molecular weight dextrans to tumor interstitium
- Validate that transient Alk5-blockade enhances retention of low molecular weight dextrans to tumor interstitium

h. Analyze evolution of leukocyte infiltration and/or protease expression in mouse mammary models using fluorescently-labeled molecular probes where animals are imaged longitudinally on weekly intervals.

Months 12-60.
- Not initiated in months 1-36

i. Select candidate molecular probes emerging from above (3.d.) that demonstrate the capability of being detected in vivo and are present at key timepoints in the evolution of breast cancer and use as a platform for development of protease-specific radiolabeled probes for single photon emission computed tomography (SPECT).

Months 12-60
- Not initiated in months 1-36

j. Demonstrate ability of agents in 3.i. to localize to tumors in proportion to the level of the specific protease targeted using in-vivo SPECT-CT imaging (X-SPECT, Xenogen Corp.) with ex-vivo autoradiography, scintillation well-counting, and immunohistochemistry to pathologically confirm levels of tissue expression and/or immune cell infiltration in areas concentrating the radiolabeled probe.

Months 24-60.
- Not initiated in months 1-36

k. Monitor relationships between presence of various immune cell populations and protease expression within mouse mammary models utilizing multi-modality imaging (MRI, SPECT-CT) in combination with the immune cell (MR) agents and protease (SPECT-CT) agents developed in 3.e. and 3.i., respectively.

Months 36-60.

- Not initiated in months 1-36

IV. REPORTABLE OUTCOMES:

A. MANUSCRIPTS:

Months 1-12 (Provided as Appendix Material in 2007 Annual Progress Report)

Tan TT, **Coussens LM**. Humoral immunity, inflammation and cancer. (2007) *Curr Opin Immunology* 19(2), 209-216

Johansson M, Tan T, de Visser KE, **Coussens LM**. (2007) Immune cells as anti-cancer therapeutic targets and tools. *J Cellular Biochemistry*, *in press. J Cellular Biochemistry*, 101: 918-926.

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Months 12-24 (Provided as Appendix Material in 2008 Annual Progress Report)

Eichten AE, Hyun WC, **Coussens LM**. (2007) Distinctive features of angiogenesis and lymphangiogenesis determine their functionality during de novo tumor development. *Cancer Research,* 67:5211-5220*.*

Kenny H, Kaur S, **Coussens LM**, Lengyel E. (2008) Adhesion of OvCa cells to peritoneum is mediated by MMP-2 cleavage of fibronectin*, J Clin Invest,* 118(4):1367-1379.

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Months 24 - 36 (COMPILED AND PROVIDED AS APPENDIX B)

DeNardo DG, Johansson M, **Coussens LM**. (2008) Inflaming gastrointestinal oncogenic programming. *Cancer Cell*, 14: 7-9.

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Affara NI, **Coussens LM.** (2008) Proteolytic pathways: Intersecting cascades in cancer development. *In:* The Cancer Degradome-Proteases and Cancer Biology, 1st Edition (Dylan Edwards, Gunilla Hoyer-Hansen, Francesco Blasi, Bonnie F. Sloane, Eds), Springer Science and Business Media, *pp 157-182.*

Affara NI, Andreu P, **Coussens LM.** (2009) Delineating protease function during cancer development. *In:* Methods in Molecular Biology, Proteases and Cancer. (Thoman Bugge and Toni Antalis, Eds.) Humana Press, *539:1-32*

Watkins GA, Jones EF, Shell MS, VanBrocklin HV, Pan MH, Hanrahan SM, Feng JJ, He J, Sounni NE, Dill KA, Contag CH, **Coussens LM**, Franc BL. (2009) Development of an optimized activatable MMP-14 targeted SPECT imaging probe. *Bioorganic & Medicinal Chemistry, 17:653-659*.

DeNardo DG, Baretto JB, Andreu P, Vasquez L, Kolhatkar N, **Coussens LM.** (2009) $CD4^+$ T cells regulate pulmonary metastasis of mammary carcinomas by enhancing protumor properties of macrophages. *Cancer Cell, in press.*

B. ABSTRACTS:

Months 1-12 **(Provided as Appendix Material in 2007 Annual Progress Report)**
Months 12-24 **(Provided as Appendix Material in 2008 Annual Progress Report)**
Months 24-36 **COMPILED AND PROVIDED AS APPENDIX C**

C. PRESENTATIONS MONTHS 24-36 (COUSSENS, LISA M):

Symposia and Workshops: **International**

2008 *7th Annual International Congress on the Future of Breast Cancer*, Kauai, Hawaii USA

2008 Cancer Research UK Cambridge Research Institute (CRI) Inaugural Annual Symposium, '*Unanswered Questions in the Tumour Microenvironment'*, Homerton College, Cambridge UK
2008 5th International Kloster Seeon Meeting, *Angiogenesis: Molecular Mechanisms and Functional Interactions*. Kloster Seeon, GERMANY
2008 **CANCER RESEARCH UK LECTURE**, NCRI Cancer Conference, Birmingham UNITED KINGDOM
2008 5th Intl Kloster Seeon Meeting on Angiogenesis, Munich GERMANY
2009 21ST Lorne Cancer Conference, Lorne AUSTRALIA
2009 6th International Symposium on the Intraductal Approach to Breast Cancer, Santa Monica CA USA
2009 **STATE-OR-THE-ART LECTURE**, International Cancer Conference, *CANCER 2009*, Dublin IRELAND

Symposia and Workshops: **National**
2008 KEYNOTE LECTURE, Fox Chase Cancer Center 13th Annual Postdoctoral Fellow and Graduate Student Symposium, Philadelphia, PA USA
2008 DOD BCRP Era of Hope Meeting 2008, Symposium Session: *Immune and Inflammatory Contributions to Breast Cancer*, AND *Era of Hope Spotlight Session*, Baltimore MD, USA
2008 *AACR Centennial Conference: Translational Cancer Medicine 2008: Cancer Clinical Trials and Personalized Medicine;* Hyatt Regency Monterey in Monterey, CA USA
2008 University of Michigan Comprehensive Cancer Center 2008 Fall Symposium, Ann Arbor MI, USA
2008 *AACR Special Conference*, *Chemical and Biological Aspects of Inflammation and Cancer*, Ko Olina Hawai, USA
2008 International Society for Biological Therapy of Cancer (iSBTc), Workshop on Inflammation in Cancer Development, Westin Horton Plaza San Diego, CA USA
2008 Skirball Symposium, New York University School of Medicine, New York, NY USA
2008 *AACR Special Conference in Cancer Research, Tumor Immunology: New Perspectives*; Miami FL, USA
2009 *1st Conference on Regulatory Myeloid Suppressor Cells*, Clearwater, FL USA
2009 Keystone Symposium, '*Extrinsic Control of Tumor Genesis*, Vancouver, British Columbia CANADA
2009 *Inflammation and Cancer: Novel Aspects of Protumor Immunity,* Major Symposium*,* 100th Annual Meeting AACR, Denver CO USA
2009 2nd Annual Retreat of the CCR-NCI Cancer and Inflammation Program, Gettysburg, PA USA

Invited Lectures/Seminars: International
2008 Institute of Cancer and the CR-UK Clinical Centre, Barts & The London School of Medicine and Dentistry, London UK
2009 University of South Hampton, UNITED KINGDOM

Invited Lectures/Seminars: National
2008 National Cancer Institute Center for Cancer Research Grand Rounds Series in Clinical and Molecular Oncology. Bethesda MD, USA
2009 University of Michigan, Oral Health Sciences Program and Biomedical Engineering Seminar Series, Ann Arbor, MI USA
2009 Department of Pharmacology, Wayne State University, Detroit, MI USA
2009 Molecular Biology Seminar Series, Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Aurora, CO USA
2009 National Institutes of Health/National Cancer Institute, Vascular Biology Seminar Series, Bethesda MD, USA

Presentations (Coussens Lab members):
Brian Ruffell, Ph.D. (Coussens lab, Postdoctoral fellow)

- '*Role of Cathepsin C During Breast Cancer Metastasis*, Brian Ruffell, Ph.D., Breast Oncology Program Retreat. San Francisco, CA. January 29-30, 2009. **Winner of "Best-in-Show Poster Presentation" Award**

- '*Immune Regulation of the Breast Tumor Microenviro*nment' Dr. Susan Love Research Foundation, Santa Monica, CA. January 16, 2009

David DeNardo, Ph.D. (Coussens lab, Postdoctoral fellow)

- *'CD4+ T Cells promote mammary tumor metastasis'*. UCSF Immunology Postdoctoral Seminal. December 2, 2008,

D. PATENTS AND LICENSES: N/A

E. DEGREES OBTAINED: N/A

F. REAGENT DEVELOPMENT:

- Generation of breeding colony of MMTV-PymT mice on the FVB/n strain background
- Generation of breeding colony of MMTV-neu mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/RAG1$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{-/-}$/CD8$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/JH$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/cathepsin C$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD8$^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/RAG1$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{+/-}$/CD8$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/JH$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/cathepsin C$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD4$^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/CD8$^{+/-}$ mice on the FVB/n strain background
- Generation of primary mammary adenocarcinoma cell line established from day 95 MMTV-PymT (FVB/n) mouse, i.e., Min#1
- Generation of primary mammary adenocarcinoma cell line established from day 76 MMTV-PymT (FVB/n) mouse, i.e., Min#2
- Generation of primary mammary adenocarcinoma cell line established from day 110 MMTV-PymT (FVB/n) mouse, i.e., O1-T1
- Generation of pulmonary metastasis of mammary adenocarcinoma cell lines established from day 110 MMTV-PymT (FVB/n) mouse, i.e., MET#2, #3, and #4
- Generation of pcHRED transfected stable subclone of MET#2 mammary adenocarcinoma pulmonary metastasis cell line established from day 95 MMTV-PymT (FVB/n) mouse, i.e., MET#2-pHcRED#1, MET#2-pHcRED#2, MET#2-pHcRED#3
- Generation of a mouse cathepsin C expression vector by insertion of murine cathepsin C cDNA into pCR2.1 vector
- Generation of breeding colony of IL4R$\alpha^{+/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/IL4R$\alpha^{-/-}$ mice on the FVB/n strain background
- Generation of breeding colony of MMTV-PymT/IL4-GFP mice on the mixed FVB/n/C57B6 strain background

G. FUNDING APPLIED FOR BASED ON WORK SUPPORTED BY ERA OF HOPE:

Months 1-12 (* indicates application was funded)

- ***DeNardo DG**, 2006. American Cancer Society Postdoctoral fellowship

Months 12-24

- Love S AND **COUSSENS LM**. 2008, CDMP/DOD, Synergistic Idea Award

Months 24-36 (* indicates application was funded; ** indicates application is pending)

- *Boudreau N and **COUSSENS LM**, 2009, NIH/NCI P50 CA58207, Bay Area Breast Cancer SPORE,

Career Development and Developmental Research Awards. Title: Macrophage-Mediated Delivery of the Breast Tumor Suppressor HoxD10 via Autologous Transfer to Breast Tumors

- ***COUSSENS LM**, Boudreau N, and Daldrup-Link H, 2008, NIH/NCI R01CA140943-01. Title: Improved Imaging and Drug Delivery Using Novel Approaches to Regulate Tissue Perfusion
- ****COUSSENS LM**, 2009, NIH/NCI P01 Title: Reprogramming immune environment in breast cancer via dendritic cells
- **COUSSENS LM**, 2008, DoD BCRP W81XWH-08-BCRP-CA, Title: Novel Approach for Enhancing Delivery of Chemotherapeutics to Breast Cancers
- **COUSSENS LM**, 2009, DoD BCRP BC087558 Era of Hope Scholar research Award, Title: Novel Strategies for Improved Diagnostic Imaging and Drug Delivery to Breast Cancers
- **COUSSENS LM, Bissell M and Weaver M, 2009** BC087522 Collaborative Innovators Award, Title: Reciprocal Interactions Between the Tumor Microenvironment, Immune Response and Metabolic State Regulate Breast Cancer Development
- ****COUSSENS LM** and Boudreau N 2009, DoD BCRP BC095507P1 Idea Award, Title: Targeted Delivery of HoxA5 and Stabilization of the Breast Tumor Microenvironment
- ***RUFFEL B,** 2008, CDMRP BCRP Postdoctoral Fellowship, Title : Role of cathepsin C during breast cancer metastasis
- **RUFFEL B,** 2008, DOD Postdoctoral Fellowship, Title : Role of cathepsin C during breast cancer metastasis
- **RUFFEL B,** 2008, National Cancer Institute of Canada Postdoctoral Fellowship, Title: Role of cathepsin C during breast cancer metastasis
- **RUFFEL B,** 2008, Canadian Institutes of Health Research Postdoctoral Fellowship, Title: Role of cathepsin C during breast cancer metastasis
- **RUFFEL B,** 2008, California Breast Cancer Research Program Postdoctoral Fellowship, Title: Role of cathepsin C during breast cancer metastasis

H. EMPLOYMENT/RESEARCH OPPORTUNITIES APPLIED FOR: N/A

V. CONCLUSION

Chronic inflammation, a pathological condition resulting from enhanced and sustained migration of leukocytes into tissue, is now regarded as a promoting force in the majority of all solid tumors in humans. When leukocytes migrate into damaged tissues, they produce a variety of soluble mediators, including growth factors and numerous proteases that promote cancer by providing growth and survival factors to initiated neoplastic cells, regulate proangiogenic programs. Moreover, it is now quite well accepted that subpopulations of these same leukocytes also possess potent proangiogenic activity as well as immune suppressive capabilities that block effective anti-tumor T cell responses. Thus, identification of the molecular and cellular pathways that regulate these two distinct pro-tumor bioactivities would reveal identification of molecules or pathway that could be targeted to neutralize the pro-growth properties of activated leukocytes, as well as those that suppress effective anti-tumor T cell immunity. Because tumor-associated myeloid cells are genetically stable cells, they are less likely to develop drug resistance than cancer cells, and drugs that inhibit selected hematopoietic cell functions should hold promise for effective anti-cancer treatments. Established tumors represent formidable opponents that harbor inherent potential for developing drug resistance. Aside from investing in earlier screening to detect and eradicate premalignant disease, our best hope for minimizing cancer is to develop combinatorial treatment strategies where intrinsic pathways regulating neoplastic cell survival are targeted, in combination with therapies effecting extrinsic pathways that neutralize pro-tumor immunity, bolster anti-tumor immunity and limit or normalize angiogenic blood vessels. Our belief is that a broader understanding of immune cells and the specific proteolytic molecules they express during will lead to development of novel anti-cancer treatments.

Previous studies from the Coussens laboratory have demonstrated that inhibition of leukocyte migration and/or leukocyte-derived proteases into hyperplastic tissue during skin carcinogenesis is sufficient to significantly decrease tumor incidence[7,42]; thus, indicating that leukocyte recruitment is a functionally significant parameter of cancer development and that inflammation may be targeted pharmacologically to affect outcome.

While we have only been experimentally addressing similar questions regarding breast carcinogenesis utilizing *in vivo* mouse and *in vitro* organotypic models for only two-years now, our compelling preliminary data indicate that indeed, mammary carcinogenesis is similarly susceptible to immuno-modulation as a therapeutic modality. We have revealed that infiltrating CD4+ T cells alter the primary mammary microenvironment in such a way that pulmonary metastasis is favored. Our data thus far indicate that infiltrating CD4+ T cells are heavily T_H2 polarized, and via their secretion of IL-4, regulate macrophage effector function that enables MECs to exit the primary tumor microenvironment and metastasize to pulmonary locales. While we do not yet know how cathepsin C plays into this response, the diminished number of pulmonary metastases that form in cathepsin C-deficient mice emanating from significantly fewer circulating malignant MECs indicates that cathepsin C is likely exerting its role also within the primary tumor microenvironment. In the next funding period, we will evaluate how CD4 T cells and cathepsin C modulate the primary tumor microenvironment and/or the microenvironment in lungs to affect metastasis. The organotypic cultures will be informative here as we can manipulate them rapidly to reveal mechanisms involved. Together, these studies will provide insight into the role adaptive immune cells play and how a leukocyte protease, e.g., cathepsin C, together regulate cancer development that will reveal a potential novel mechanism with which to target tumor cells with anti-cancer therapeutics. While our novel SPECT RDA molecular probes have been validated in vivo their likely translation to a clinical tool is limited; thus, we have initiated studies to evaluate if primary monocytes can be utilized as noninvasive imaging reagents. Our pilot studies indicate this is a feasible approach. We will pursue this route of investigation to reveal if CD4 T cells and/or selective populations of myeloid cells can similarly be labeled and imaged noninvasively following their appropriate trafficking back to either primary or metastatic tumors. These studies will be bolstered by enhanced delivery of high molecular weight imaging compounds following transient Alk5-blockade, and targeting of contrast agents and/or cell-based therapies tumors.

VI. BIBLIOGRPHY:

1 Balkwill, F. and Mantovani, A., Inflammation and cancer: back to Virchow? *Lancet* **357** (9255), 539 (2001).

2 Balkwill, F., Charles, K. A., and Mantovani, A., Smoldering and polarized inflammation in the initiation and promotion of malignant disease. *Cancer Cell* **7** (3), 211 (2005); Johansson, M., Denardo, D. G., and Coussens, L. M., Polarized immune responses differentially regulate cancer development. *Immunol Rev* **222**, 145 (2008).

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[40] Choi, H. et al., Iron oxide nanoparticles as magnetic resonance contrast agent for tumor imaging via folate receptor-targeted delivery. *Acad Radiol* **11** (9), 996 (2004).

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VII. APPENDICES

A. Complete academic Curriculum vitae for Dr. Lisa M. Coussens
B. Collected publications from Months 24-36
C. Collected Abstracts from Months 24 - 36

APPENDIX A

CURRICULUM VITAE

June 2009

Lisa M. Coussens, Ph.D.
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Department of Pathology
University of California, San Francisco
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http://cancer.ucsf.edu/coussens/index.php

I. EDUCATION:

1976 - 1980	San Francisco State University	B.A.	Biology
1988 - 1993	University of California, Los Angeles	Ph.D.	Biological Chemistry
1993 - 1997	University of California, San Francisco	Post-Doctoral Fellow	Cancer Biology

II. PRINCIPAL POSITIONS HELD:

1981 - 1988	Genentech, Inc., South San Francisco	Research Associate	Molec. & Devel. Biology
1997 - 1999	University of California, San Francisco	Assistant Research Biochemist	Hormone Research Inst.
1999 - 2004	University of California, San Francisco	Assistant Professor, In Residence	Cancer Research Inst. & Dept of Pathology
2004 - 2006	University of California, San Francisco	Associate Professor, In Residence	Cancer Research Inst. & Dept. of Pathology
2006 - 2007	University of California, San Francisco	Associate Professor	Dept. of Pathology & Cancer Research Inst.
2007 - present	University of California, San Francisco	Professor	Dept. of Pathology & Cancer Research Inst.

OTHER POSITIONS HELD CONCURRENTLY:

1989 - 1992	Whittier College, Whittier, CA	Lecturer	Biology Dept
1992	Genentech, Inc., South San Francisco	Scientific Consultant	Dept. of Legal Affairs

III. HONORS AND AWARDS:

1985	Recognition Award	Genentech, Inc.,
1986	Recognition Award	Genentech, Inc.,
1988	Recognition Award	Genentech, Inc.
2000 - 02	Hellman Family Award For Early Career Faculty	Univ. of Calif., San Francisco
2000 - 01	V Foundation Scholar	The V Fndt. for Cancer Research
2000 - 03	Edward Mallinckrodt, Jr. Fndt. Award for Medical Research	Edward Mallinckrodt, Jr. Fndt.
2002	Gertrude B. Elion Cancer Research Award	Am. Assoc. for Cancer Research
2006 - 11	Era of Hope Scholar Award	Department of Defense, Breast Cancer Research Program

IV. PROFESSIONAL ACTIVITIES

1999 - present	Member	Graduate Program in BioMedical Sciences (BMS)	Univ. of Calif., San Francisco
1999 - present	Member	Helen Diller Family Comprehensive Cancer Center	Univ. of Calif., San Francisco
2000 - present	Member	Graduate Program in Biological Sciences (PIBS)	Univ. of Calif., San Francisco
2001 - present	Co-Director	Mouse Pathology Core	Helen Diller Family Comprehensive Cancer Center, Univ. of Calif., San Francisco
2004 – present	Member	Program in Immunology	Univ. of Calif., San Francisco
2004 – 2007	Senior Editor	***Cancer Research (****Cell, Tumor and Stem Cell Biology* Section)	American Association Cancer Research
2007 – present	Deputy Editor	***Cancer Research***	American Association Cancer Research
2007 – present	Senior Editor	***Cancer Research (****Tumor Microenvironment* Section)	American Association Cancer Research
2007 - 2010	Member	External Scientific Advisory Board, University of Minnesota Cancer Center	University of Minnesota Cancer Center
2007 – 2011	Member	External Scientific Advisory Board, CA U54: *Aging, Tumor Microenvironment and Prostate Cancer*; P.I. Steve Plymate, Univ. of Washington, HMC.	University of Washington
2008 – 2011	Member	Board of Directors (*elected*)	American Association of Cancer Research
2009 - present	Member	External Scientific Advisory Board; Neuroblastoma Program Project Grant	Children's Hospital Los Angeles, Univ. of Southern California

National and International Meetings Organized:

2005 Keystone Symposia, *Inflammation and Cancer*, Co-organizer with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckinridge, CO, USA

2006 5th Annual Timberline Symp. on Epithelial Cell Biology, '*Intrinsic and Microenvironmental Regulation of Epithelial Cancer'*, Co-Organizer with Dr. Harold Moses (Vanderbilt University, TN, USA), Timberline, OR, USA

2007 Keystone Symposia, *Inflammation and Cancer*, Co-Organizer with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA); Santa Fe, New Mexico, USA

2008 AACR Special Conference: *Inflammation and Cancer*, Co-organizer with with Drs. Michael Karin and Larry Marnett. Oahu, Hawaii, USA.

2008 International Society for Biological Therapy of Cancer (iSBTc), *2008 Workshop on Inflammation in Cancer Development*, Co-Organizer with Drs. Michael Karin, (UCSD), Steven Dubinett (UCLA), and George Weiner (WU); San Diego CA USA

V. PROFESSIONAL ORGANIZATIONS

Memberships

2000 - 2009	American Society for Matrix Biology
2000 - present	American Association for Cancer Research
2001 – 2008	American Society for Cell Biology
2004 - 2009	American Society for Investigative Pathology
2004 - 2009	International Protease Society

Service to Professional Organizations

American Association for Cancer Research

2003	Subsection Co-chair (Tumor Progression, Invasion and Metastasis) Cellular, Molecular and Tumor Biology Subcommittee, AACR Program Committee for *94th Annual Meeting*.
2003	Chair and organizer, Educational Session *(*Proteases: Successes and Failures): *94th Annual Meeting*, Washington D.C., USA
2003	Minisymposium Co-chair (Inflammatory Mediators & Cancer): *94th Annual Meeting*, Washington D.C., USA
2004 - 2006	Member, Grants Committee
2005	Minisymposium Co-Chair (Inflammation, Microenvironment and Tumor Progression): *96th Annual Meeting*, Anaheim, CA USA
2005	Session Chair (Inflammation*)*: *AACR Special Conference: Cancer, Proteases and the Microenvironment*, Bonita Springs, Florida. USA
2006	Subsection Co-chair (Tumor Progression, Invasion and Metastasis) of the Tumor Biology Subcommittee, AACR Program Committee for *97th* Annual meeting
2006	Minisymposium Co-Chair (Inflammation and Cancer): *97th Annual Meeting*, Washington DC, USA
2006	Co-Chairperson, Program Committee: *6th Annual Frontiers in Cancer Prevention Research Conference*, December 5-8, 2007, Philadelphia, PA USA.
2006-2009	Steering Committee Member: AACR Tumor Microenvironment Working Group (TME/AACR).
2007	Organizer, Education session (Inflammation and Cancer), *98th Annual Meeting*, Los Angeles, USA
2007	Minisymposium Co-Chair (Tumor Microenvironment): *98th Annual Meeting*, Los Angeles, CA USA
2007	Co-Chairperson, Program Committee: *2008 99th Annual Meeting of the AACR*. April 12-16, 2008, San Diego, CA. USA
2008	Program Committee Member, Tumor Microenvironment Subcommittee for *99th Annual Meeting of the AACR*. April 12-16, 2008, San Diego, CA. USA
2007-2010	Member, AACR Special Conferences Committee
2008	Co-Organizer Special Conference: *Inflammation and Cancer*, with Drs. Michael Karin and Larry Marnett. Oahu, Hawaii, USA.
2008-2011	Member, Board of Directors (elected)
2009	Member, 2009 Education Committee, 2009 100th AACR Annual Meeting, Denver, CO
2009	Organizer and Chair: *Inflammation and Cancer: Novel Mechanisms Regulating Protumor Immunity* Major Symposium, 2009 100th AACR Annual Meeting, Denver, CO
2009	Organizer and Chair: Education Session, *Aspects of the Tumor Microenvironment that Regulate Solid Tumor Development*, 2009 100th AACR Annual Meeting, Denver, CO
2010	Co-Chairperson, Program Committee: *2010 101st Annual Meeting of the AACR*, April 17-21, 2010, Washington, DC USA
2009	Member, Scientific Review Committee for *Stand Up to Cancer Innovative Research Grants*

American Society for Cell Biology

2000 American Society for Cell Biology, photo credits in '*Exploring the Cell'* Ed. W. Wells
2001 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee, 40th Annual Meeting, Washington, DC, USA
2001 Co-chair and Co-organizer, Mini-symposium (Microenvironment/Extracellular Matrix in Development and Disease): 40th Annual Meeting, Washington, DC, USA
2003 Table Leader, Career Discussion Lunch, Women in Cell Biology and Education Committee of the ASCB, *42nd Annual Meeting*, San Francisco, CA, USA
2006 Co-Chair Minisymposium (Cancer Mechanisms*)*: *46th Annual Meeting*, San Diego CA, USA

American Cancer Society

1999 14th Annual Excalibur Round Table, San Francisco, CA, USA
2000 San Mateo County Annual Volunteer Meeting, San Mateo, CA, USA

International Society for Preventive Oncology

2002 Session Chair (Chemoprevention*)*: *6th Annual Meeting*, Pasteur Institute, Paris, France.
2002 Poster Judge (Chemoprevention*)*: *6th Annual Meeting*, Pasteur Institute, Paris, France.

International Proteolysis Society

2007 Member, International Scientific Advisory Committee, 5th General Meeting of the International Proteolysis Society, Rion-Patras, GREECE.

International Society for Biological Therapy of Cancer

2008 Co-Organizer, *2008 Workshop on Inflammation in Cancer Development*, San Diego CA, USA

Service to Professional Publications:

2003 - 2005	Associate Editor, ***Cancer Research***
2005 – 2007	Editorial Board, ***Carcinogenesis***
2004 – 2007	Senior Editor, ***Cancer Research (****Cell, Tumor and Stem Cell Biology* Section)
2007 – present	Senior Editor, ***Cancer Research (****Tumor Microenvironment* Section)
2007 – present	Deputy Editor, ***Cancer Research***
2007	Guest Editor, PNAS Editorial Board
2008	Guest Editor (with Tyler Jacks), Current Opinion in Genetics & Development
2008 – present	Editorial Board, ***Cancer Microenvironment***

Ad hoc reviewing

1994 Oncogene;
1995 Am J Pathology; Matrix Biology; J Cell Biology
1999 Am J Pathology; Cancer Letters; Nature Medicine; Nature; PNAS; Cell Motility & the Cytoskeleton; Cancer Research
2000 Am J Pathology; Cancer Research; Genes & Development; Int. J Cancer
2001 J Cell Biology; Int. J of Cancer; EMBO; Neoplasia; Cancer Research
2002 Cancer Research; Am J Pathology; Int. J Cancer; Biological Chemistry; Cancer Cell; Cancer Letters
2003 PNAS; Cancer Research; Int. J of Cancer; J Molecular Medicine; Biological Chemistry; Science; Cancer Cell; Nature Medicine; J Leukocyte Biology; Neoplasia; Am J Pathology
2004 Lancet; Cancer Cell; Cancer Research; American J Pathology; J Cell Biology; Nature Reviews Immunology; Nature Reviews Cancer; PNAS; J Biological Chemistry; Nature; J Exp Med; Int J Cancer

2005 Nature Medicine, Cancer Cell, Cancer Research; Am J Pathology; Cell; Nature; Nature Reviews Immunology; Nature Reviews Cancer; Carcinogenesis
2006 Nature Reviews Cancer; Nature; Nature Medicine; Cell; Cancer Research; Clinical Cancer Research; J Exp Med; Cancer Cell: Am J Pathology; J Cell Biology
2007 Cell; Nature; PNAS: J Cell Biology; Cancer Research; J Exp Med; Breast Cancer Research
2008 Cancer Cell; PNAS; J Immunology; Nature; J Exp Med
2009 Cancer Cell; Cell; Nature; J Exp Med; J Clin Invest, Cancer Research,

VI. INVITED PRESENTATIONS

Symposia and Workshops: International

1996 *Human Tumor Heterogeneity II*: Cytometric Measurement of Growth Regulation and Genetic Alterations: International Society of Analytical Cytometry. Kananaskas, Alberta, Canada.
1997 *GeneMedicine-Boehringer Mannheim Cancer Alliance: Technology Workshop*. Cancún Mexico.
2001 *2nd Annual International Protease Society*. Freising, Germany.
2002 *6th International Symposium on Predictive Oncology & Intervention Strategies*, Pasteur Institute, Paris, France
2002 **KEYNOTE LECTURE**, *Dutch Cancer Society Annual Symposium*, Luntern, The Netherlands
2002 **KEYNOTE LECTURE**, *Cancer: Genome, Signal & Environment, Takeda Genome Urology International*, Kyoto, Japan
2003 *2nd Annual International Symposium on Epithelial Biology*, Timberline, Oregon USA
2004 10th International Congress of the *Metastasis Research Society*, 'Progress Against Tumor Progression', Genoa Italy
2005 *2005 International Consortium Meeting of the Children's Tumor Foundation: Molecular Biology of NF1, NF2 and Schwannomatosis,* Aspen, CO, USA
2005 *International Symposium on Systems Genome Medicine - Bench to Bedside,* Institute of Medical Sciences University of Tokyo, Tokyo, Japan
2005 *Immunotherapy of Cancer*, XI Annual Symposium of the Danish Cancer Society, Copenhagen, Denmark
2005 *4th General Meeting of the International Proteolysis Society*, Quebec City, Canada
2006 Centro Nacional de Investigaciones Oncológicas (CNIO) Cancer Conference: *Inflammation and Cancer*, Mardid SPAIN
2006 18th Annual Pezcoller Symposium *'Tumor Microenvironment: Heterotypic Interactions'*, Trento ITALY
2006 European Association for Cancer Research (EACR) 1st Annual Meeting, Budapest HUNGARY
2006 XXXIVth Meeting of the International Society for Oncodevelopmental Biology and Medicine (ISOBM: Tumor Biology, Detection and Therapy, Pasadena, CA, USA
2006 37th International Symposium of the Princess Takamatsu Cancer Research Fund '*Cancer Cells and Their Microenvironment*', Tokyo, JAPAN
2007 *4th International Conference on Tumor Microenvironment*, Florence, ITALY
2007 *2nd International Symposium on Cancer Metastasis and the Lymphovascular System*: Basis for Rational Therapy, San Francisco CA USA
2007 CNIO – Nature Symposium on "*Oncogenes and Human Cancer*". The Next 25 Years", Madrid SPAIN
2007 **KEYNOTE LECTURE**, *7th International Symposium on Hodgkin Lymphoma*, Cologne, GERMANY
2007 **CANDLELIGHT LECTURE**, *Inflammation and Cancer: From molecular links to bed side*; Inaugural meeting for the *Istituto Clinico Humanitas*, Milan ITALY
2008 *7th Annual International Congress on the Future of Breast Cancer*, Kauai, Hawaii USA

2008 Cancer Research UK Cambridge Research Institute (CRI) Inaugural Annual Symposium, '*Unanswered Questions in the Tumour Microenvironment'*, Homerton College, Cambridge UK
2008 5th International Kloster Seeon Meeting, *Angiogenesis: Molecular Mechanisms and Functional Interactions*. Kloster Seeon, GERMANY
2008 **CANCER RESEARCH UK LECTURE**, NCRI Cancer Conference, Birmingham UNITED KINGDOM
2008 5th Intl Kloster Seeon Meeting on Angiogenesis, Munich GERMANY
2009 21ST Lorne Cancer Conference, Lorne AUSTRALIA
2009 6th International Symposium on the Intraductal Approach to Breast Cancer, Santa Monica CA USA
2009 **STATE-OR-THE-ART LECTURE**, International Cancer Conference, *CANCER 2009*, Dublin IRELAND

UPCOMING INVITATIONS
2009 19th Annual BioCity Symposium, 'Tumor Microenvironment in Cancer Progression", Tirku FINLAND
2009 **KEYNOTE LECTURE**, European Association of Cancer Research, Special Conference on *Inflammation and Cancer*, Berlin GERMANY
2009 7th International Symposium on Minimal Residual Cancer, Athens, GREECE
2009 13th World Conference of Lung Cancer, San Francisco, CA USA
2009 Tri-Society Annual Conference of the Society for Leukocyte Biology, International Cytokine Society, and the International Society for Interferon and Cytokine Research, Lisbon, Portugal
2009 5th International Conference on Tumor Microenvironment, Versailles, FRANCE
2009 **PRESIDENT'S PLENARY SESSION**: Italian Cancer Society Annual Meeting, Milano ITALY
2010 Spetsai Summer School 2010: *From Pluripotency to Senescence: Molecular Mechanisms of Development, Disease and Ageing*. Spetsai, GREECE

Symposia and Workshops: National
1994 *Current Transgenic Technology*, B & K Universal, San Mateo, CA, USA
1997 *Biology of Proteolysis*, Cold Spring Harbor Laboratory, NY, USA
1997 *Molecular Biology & Pathology of Neoplasia*, AACR, Keystone, CO, USA
1997 *Matrix Metalloproteinases*, Gordon Research Conference, Proctor Academy, New London, NH, USA
1998 *Proteolysis*, Gordon Research Conference, Colby-Sawyer College, New London, NH, USA
1998 *Cellular Targets of Viral Carcinogenesis*, AACR Special Conference. Dana Point, CA, USA
1998 *Mechanisms of Tumor Growth & Invasion Mediated by Proteolysis*, UCSF-Molecular Design Institute. San Francisco, CA, USA
1999 *Tumor Microenvironment*, Education Session, AACR Annual Meeting. Philadelphia, PA, USA
1999 *Matrix Metalloproteinases*, Gordon Research Conference, Colby-Sawyer New London, NH, USA.
2000 *Epithelial-Stromal Interactions & Tumor Progression Workshop*, National Cancer Inst., Bethesda, MD, USA
2000 10th National Conference of the Inflammation Research Association, Hot Springs, VA, USA
2001 *'Meet-the-Expert' Sunrise Session*, AACR Annual Meeting, New Orleans, LA, USA
2002 *Chemotherapy of Experimental & Clinical Cancer*, Gordon Research Conference, Colby Sawyer College, New London, NH, USA
2002 *Proteolytic Enzymes & their Inhibitors*, Gordon Research Conference, Colby Sawyer, New London, NH, USA
2002 *From the Cancer Cell to a Tumor - Tumors as Outlaw Organs*, Schilling Research Conference, The American Cancer Society, Aptos CA, USA
2002 *Cancer Intervention 2002*, Van Andel Research Institute, Grand Rapids, Michigan USA
2002 *Pathobiochemistry B Study Section Workshop*, Natl. Cancer Institute, Hilton Head, SC, USA

2002 *Proteases, Extracellular Matrix and Cancer,* AACR Special Conference, Hilton Head Island, SC, USA
2002 *ECM and Cancer*, Minisymposium, ASCB Annual Meeting, San Francisco, CA, USA
2003 *Matrix Metalloproteinases*, Gordon Research Conference, Big Sky, Montana, USA
2003 *Angiogenesis & Microcirculation*, Gordon Research Conference, Salve Regina, Newport R.I., USA
2003 *Inflammatory Cells and Cancer,* Symposium, American Society of Hematology 2003 Annual Meeting, San Diego, CA, USA
2003 *Validation of a Causal Relationship: Criteria to Establish Etiology*, National Cancer Institute, Cancer Etiology Branch, Washington, DC, USA.
2003 *Functional Imaging of Proteolysis*, Special Session, ASCB Annual Meeting, San Francisco, CA, USA
2004 Scleroderma Research Foundation Annual Scientific Workshop, San Francisco, CA, USA
2004 *Systems Biology of Cancer: The Tumor as an Organ,* Symposium, 95th AACR Annual Meeting. Orlando, FL, USA
2004 *Inflammation and Cancer,* Symposium, 95th AACR Annual Meeting. Orlando, FL, USA
2004 *Remarkable Role of the Microenvironment in Development and Disease Pathogenesis,* Symposium; Experimental Biology 2004, Sponsored by: the Assoc. of Anatomy, Cell Biology and Neurobiology, Washington, D.C., USA.
2004 *Molecular and Cellular Basis of Disease: Structure and Function of the Extracellular Matrix in Disease: Novel Roles and Regulation of MMPs and TIMPs in Disease,* Symposium; Experimental Biology 2004, Sponsored by: the Am. Society of Investigative Pathology, the American Society for Matrix Biology and the North American Vascular Biology organization. , Washington, D.C., USA.
2004 Pacific Coast Protease Workshop, Half Moon Bay, CA, USA.
2004 19th Aspen Cancer Conference: *Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy.* Aspen, CO, USA.
2005 Keystone Symposia, *The Role of Microenvironment in Tumor Induction and Progression (J5),* Banff, Alberta CANADA
2005 Keystone Symposia, *Inflammation and Cancer (B8),* Breckenridge, CO, USA
2005 *Symposium on Inflammation, Repair and Carcinogenesis in Liver, Pancreas and Colon.* UCSF Liver Center and the Program in Gastrointestinal Cancer of the UCSF Cancer Center, Rohnert Park, CA, USA
2005 *In the Forefront of Advances in Cancer Research,* Symposium, 96th AACR Annual Meeting. Anaheim, CA, USA
2005 *Macrophage Symposium*, AMGEN, Seattle, WA, USA
2005 *Immune Response to Cancer Symposium*, 41st Annual Meeting, American Society Clinical Oncology (ASCO), Orlando. FL. USA
2005 *Phagocyte*, Gordon Research Conference, New London, CT, USA
2005 *Mouse Models of Human Cancer Consortium*, Annual Steering Committee Meeting, New Brunswick, NJ USA
2005 *Matrix Metalloproteinases*, Gordon Research Conference, Big Sky, Montana, USA
2005 *Annual Buffalo Regional Conference on Immunology*, Buffalo, NY, USA
2005 2005 Montagna Symposium on *'Tissue repair - molecular mechanisms and clinical challenges'*, Salishan Lodge, OR, USA
2005 4th Annual AACR Conference on *Frontiers in Cancer Prevention Research*, Baltimore MD, USA
2005 AACR Special Conference, *Cancer, Proteases and the Microenvironment*, Bonita Springs, Florida. USA
2006 Timberline Annual Symposium on Epithelial Biology, *Intrinsic and Microenvironmental Regulation of Epithelial Cancer*', Timberline Lodge, Oregon, USA

2006 Keystone Symposium, *Molecular Targets for Cancer Prevention*, Granlibakken Resort, Tahoe City, CA, USA

2006 *Inflammation and Cancer*, Symposium, *97th AACR Annual Meeting*. Washington, D.C., USA

2006 Lineberger Cancer Center's *30th Annual Scientific Symposium*, University of North Carolina, Chapel Hill, North Carolina, USA

2006 KEYNOTE LECTURE, *Vanderbilt-Ingram Cancer Center Retreat 2006*, Vanderbilt University, Nashville TN, USA

2006 Tumor Biology Plenary Lecture, *Advances in Neuroblastoma Research 2006*, Los Angeles, CA, USA

2006 *Genetic, Cellular and Microenvironmental Determinants of Tumor Progression and Metastasis: A 'TPM' Workshop Honoring Martin L Padarathsingh, Ph.D.* TPM Study Section Workshop, Natl. Cancer Institute, Georgetown, VA, USA

2006 ASCO/Federation of European Societies Symposium: *Inflammation in Cancer Progression*, 2006 ASCO Annual Meeting, Atlanta, GA, USA

2006 AACR Special Conference, *Mouse Models of Cancer*, Cambridge, MA, USA

2006 AACR Special Conference, *Tumor Immunology: An Integrated Perspective.* Miami, FL, USA

2007 7th AACR-Japanese Cancer Association Joint Conference: *In the Forefront of Basic and Translational Cancer Research*, Waikoloa, Hawaii, USA

2007 Keystone Symposium, *'Mouse Models at the Frontiers of Cancer Discovery'*, Whistler, British Columbia, CANADA

2007 Keystone Symposium '*Inflammation and Cancer'*, Santa Fe, NM, USA

2007 AAAS Annual Meeting, *Healthy Aging: Inflammation and Chronic Diseases'* Symposium, San Francisco, CA USA

2007 Tumor Microenvironment and Tumor-Stromal Interactions Workshop: Sponsored by Biogen Idec Inc., Oncology Discovery Research, San Diego CA USA

2007 American Thoracic Society 2007 International Conference, *San Francisco Science: Inflammation, Immunity and Signaling*. San Francisco, CA USA

2007 22nd Aspen Cancer Conference: Mechanisms of Toxicity, Carcinogenesis, Cancer Prevention and Cancer Therapy, Aspen CO, USA

2007 Gordon Research Conference, *Epithelial Differentiation & Keratinization*, Bryant University, Smithfield, RI, USA

2007 AACR, *Frontiers in Cancer Prevention Research Conference*, Philadelphia, PA, USA

2007 National Cancer Institute Workshop, '*Profiling of Immune Response to Guide Cancer Diagnosis, Prognosis and Prediction of Therapy'*, Bethesda, MD, USA

2008 47th Midwinter Conference of Immunologists, '*Meeting the challenge: Immunobiology in health and disease*', Asilomar, CA USA

2008 AACR-TREC-NCI Conference on *Energy Balance and Cancer: Mediators and Mechanisms*, Lansdowne, VA USA

2008 Keystone Joint Symposium, '*Cell Death in the Immune System / Cell Death and Cellular Senescence'*, Beaver Run Resort in Breckenridge, CO, USA

2008 Keystone Symposium, '*Inflammation, Microenvironment and Cancer'*, Snowbird Resort in Snowbird, Utah, USA

2008 The John F. Anderson Memorial Lectures in Medicine, '*The Linkage between Inflammation and Cancer*', University of Virginia, Charlottesville VA, USA

2008 *Tumor Microenvironment Symposium*, Stony Brook University, Stony Brook. NY. USA

2008 KEYNOTE LECTURE, Fox Chase Cancer Center 13th Annual Postdoctoral Fellow and Graduate Student Symposium, Philadelphia, PA USA

2008 DOD BCRP Era of Hope Meeting 2008, Symposium Session: *Immune and Inflammatory Contributions to Breast Cancer*, AND *Era of Hope Spotlight Session*, Baltimore MD, USA

2008 *AACR Centennial Conference: Translational Cancer Medicine 2008: Cancer Clinical Trials and Personalized Medicine;* Hyatt Regency Monterey in Monterey, CA USA
2008 University of Michigan Comprehensive Cancer Center 2008 Fall Symposium, Ann Arbor MI, USA
2008 *AACR Special Conference, Chemical and Biological Aspects of Inflammation and Cancer*, Ko Olina Hawai, USA
2008 International Society for Biological Therapy of Cancer (iSBTc), Workshop on Inflammation in Cancer Development, Westin Horton Plaza San Diego, CA USA
2008 Skirball Symposium, New York University School of Medicine, New York, NY USA
2008 *AACR Special Conference in Cancer Research, Tumor Immunology: New Perspectives*; Miami FL, USA
2009 *1st Conference on Regulatory Myeloid Suppressor Cells*, Clearwater, FL USA
2009 Keystone Symposium, '*Extrinsic Control of Tumor Genesis*, Vancouver, British Columbia CANADA
2009 *Inflammation and Cancer: Novel Aspects of Protumor Immunity,* Major Symposium, 100th Annual Meeting AACR, Denver CO USA
2009 2nd Annual Retreat of the CCR-NCI Cancer and Inflammation Program, Gettysburg, PA USA

UPCOMING INVITATIONS
2009 24th Annual Aspen Cancer Conference, Aspen, CO, USA
2009 AACR Special Conference, Advan*ces in Breast Cancer Research: Genetics, Biology, and Clinical Applications*, San Diego CA USA
2009 2009 Geoffrey Beane Cancer Research Symposium: *Inflammation and Cancer*, Memorial-Sloane Kettering Cancer Center, New York NY USA
2010 Joint Keystone Symposia, *Role of Inflammation in Oncogenesis/Molecular and Cellular Biology of Immune Escape in Cancer*, Keystone CO USA

Invited Lectures/Seminars: International

2000 Medical Genome Center, Division of Molecular Medicine, Australian National University, Canberra, A.C.T. Australia.
2001 German Cancer Center, Heidelberg, Germany.
2001 MERCK Pharmaceutical, Damstedt Germany.
2003 University of Toronto, Ontario Cancer Institute & Princess Margaret Hospital, Toronto, Ontario, CANADA
2004 Cancer Research UK, Barts & The London Queen Mary's School of Medicine & Dentistry, John Vane Science Center, Charterhouse Square, London, UK
2004 Cancer Research UK, London Research Institute, Lincoln's Inn Fields Laboratories, London, UK
2004 University of British Columbia, Department of Biochemistry and Molecular Biology, Vancouver, British Columbia, Canada
2007 Angiogenesis and Tumor Targeting Research Unit & Telethon Institute for Gene Therapy, San Raffaele Scientific Institute, Milan, ITALY
2008 Institute of Cell Biology, ETH Zurich Switzerland
2008 Institute of Cancer and the CR-UK Clinical Centre, Barts & The London School of Medicine and Dentistry, London UK
2009 University of South Hampton, UNITED KINGDOM

UPCOMING INVITATIONS
2009 The Netherlands Cancer Institute, Amsterdam, THE NETHERLANDS
2009 Instituto Tumori, Milano ITALY

Invited Lectures/Seminars: National

1997 Biologic Therapy Research Conference. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1997 Immunology Seminar Series. Univ. of Pittsburgh Medical Center, Pittsburgh, PA, USA
1999 Axys Pharmaceuticals, South San Francisco, CA, USA
1999 Berlex Pharmaceuticals, Emeryville, CA, USA
1999 Axys Pharmaceuticals, La Jolla, CA, USA
1999 14th Annual Excalibur Round Table, American Cancer Society, San Francisco, CA, USA
1999 Colloquium in Microbiology, Cell and Molecular Biology. San Francisco State Univ., San Francisco, CA, USA
2000 Chiron Corporation, Emeryville, CA, USA
2000 Oral and Pharyngeal Cancer Branch/NIDCR, National Institutes of Health, Bethesda, MD, USA
2000 Fibrogen, Inc., South San Francisco, CA, USA
2000 Scios Inc., Sunnyvale, CA, USA
2000 Molecular Biology Department, University of Southern California, Los Angeles, CA, USA
2001 Dept. of Pediatric Hematology and Oncology, Children's Hospital Los Angeles, Univ. of Southern California, Los Angeles, CA, USA
2001 Jonnson Comprehensive Cancer Center, Univ. of Calif., Los Angeles, Los Angeles, CA, USA
2002 Institute for Engineering and Medicine, Univ. of Pennsylvania, Philadelphia, PA, USA
2002 Oncology Grand Rounds, Univ. of Missouri, Columbia, MO.
2002 Cancer Center, Univ. of California, Davis, Davis CA, USA
2002 AstraZeneca, Waltham, MA USA
2002 Pharmacology Seminar Series, Dept. of Pharmacology, Wayne State Univ., Detroit, MI, USA
2003 Dept. of Biology, Univ. of Calif., San Diego, San Diego, CA USA
2003 Tularik, Inc., South San Francisco, CA USA
2003 Dept. of Cancer Biology's Cancer Metastasis Research Program Seminar Series, M.D. Anderson Cancer Center, Univ. of Texas, Houston, TX, USA
2003 Dept. of Cancer Biology, Stanford University, Stanford, CA, USA
2004 Burnham Cancer Institute, San Diego, CA, USA
2004 The Wistar Cancer Institute, Philadelphia, PA, USA
2004 Regeneron Pharmaceuticals, Inc. Tarrytown, New York, USA
2004 Keynote Lecture: Vanderbilt University Digestive Disease Research Center Retreat, Vanderbilt University, Nashville, TN, USA
2004 Dana Farber Cancer Center, Harvard Medical School, Boston MA, USA
2004 Indiana University, Herman B. Wells Center for Pediatric Research and Clinical Cancer Center, Indianapolis IN, USA
2004 Immunology Graduate Program Seminar, Stanford University, Stanford, CA, USA
2005 Dept. of Nutritional Sciences & Toxicology, Univ. of Calif., Berkeley, Berkeley, CA USA
2005 Rigel, Inc., South San Francisco, CA USA
2005 Dept of Pathology & Lab Medicine, Univ. of California, Los Angeles, Los Angeles, CA USA
2006 Division of Cancer Biology and Angiogenesis in the Department of Pathology at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA USA
2006 Department of Molecular and Medical Pharmacology, University of California, Los Angeles, Los Angeles, CA USA
2007 *Lymphoma and Myeloma Conference,* M.D. Anderson Cancer Center, Houston, TX, USA
2007 University of Minnesota, Dept. of Lab Medicine and Pathology, Minneapolis, MN, USA
2007 Memorial-Sloan Kettering Cancer Center, Program in Cancer Biology and Aging, New York NY, USA
2007 Abramson Family Cancer Research Institute and Univ. of Pennsylvania, Division of Hematology-Oncology, Philadelphia, PA USA

2007 Albert Einstein College of Medicine, New York NY, USA
2007 Oncology Division Research, Biogen Idec Inc., San Diego, CA USA
2007 Genentech, Inc. Immunology Program. South San Francisco, CA USA
2007 University of Iowa Carver College of Medicine, Dept of Pathology, *Pathology Grand Rounds*, Iowa City, Iowa, USA
2007 Hollings Cancer Center, Medical University of South Carolina, Charleston, SC, USA
2007 University of Michigan, Program in Immunology and Cancer Research Series, Ann Arbor, MI USA
2008 Department of Pathology/UCLA School of Medicine Seminar, Los Angeles CA USA
2008 ANNUAL KEYNOTE LECTURE, Dept of Cancer Biology, Meharry Medical College, Nashville, TN USA
2008 University of California, Davis Cancer Center, Sacramento, CA USA
2008 Department of Immunology, University of Pittsburgh School of Medicine. Pittsburgh, PA, USA
2008 Cancer Biology Series, Ben May Cancer Center, University of Chicago, Chicago, IL, USA
2008 National Cancer Institute Center for Cancer Research Grand Rounds Series in Clinical and Molecular Oncology. Bethesda MD, USA
2009 University of Michigan, Oral Health Sciences Program and Biomedical Engineering Seminar Series, Ann Arbor, MI USA
2009 Department of Pharmacology, Wayne State University, Detroit, MI USA
2009 Molecular Biology Seminar Series, Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Aurora, CO USA
2009 National Institutes of Health/National Cancer Institute, Vascular Biology Seminar Series, Bethesda MD, USA

UPCOMING INVITATIONS
2009 Genentech, Inc. Immunology Program. South San Francisco, CA USA
2009 Breast Cancer Network of Strength, California Breast Cancer Organizations, Northern California Affiliate, David CA USA
2010 Cold Spring Harbor Laboratory, CSH NY USA
2010 Department of Cell Biology & Physiology Washington University, St Louis, MO USA

Invited Lectures/Seminars: UCSF
1997 Breast Cancer SPORE Seminar. UCSF
1999 Cancer Research Institute Retreat, Tomales Bay, CA
2000 Chemistry and Cancer: How Chemistry-Based Tools Are Helping Solve Today's Serious Health Problems, Dev. & Alumni Relations, UCSF
2000 Oncology Grand Rounds, Department of Hematology and Oncology, UCSF
2000 PIBS-Cell Biology Seminar Series, UCSF
2000 Pathology and Lab Medicine Grand Rounds, UCSF
2000 BMS Student Pizza Talk, UCSF
2000 Cell Cycle & Dysregulation Club, Comprehensive Cancer Center, UCSF
2000 Comprehensive Cancer Center Retreat, Granlibakken, Tahoe City, CA
2001 BMS Student Pizza Talk, UCSF
2001 Pathology and Lab Medicine Grand Rounds, Departments of Medicine and Pathology, UCSF
2001 UCSF, Cell Biology Retreat, Wilbur Hot Springs, CA, USA
2001 UCSF TETRAD Retreat, Granlibakken, Lake Tahoe, CA, USA
2001 UCSF Cancer Research Institute/BMS Retreat, Granlibakken, Lake Tahoe, CA. USA
2002 Current Topics in Medical Science, UCSF Medical Scientist Training Program (M170.09)
2002 Mouse Models of Human Cancer Program, Comprehensive Cancer Center, UCSF

2002 Cancer Research Institute Retreat, Santa Cruz, CA
2003 PIBS Student Pizza Talk, UCSF
2003 Breast Oncology Program, Comprehensive Cancer Center, UCSF
2003 Comprehensive Cancer Center Faculty Retreat: *Identification and Functional Assessment of Cancer Effectors*, Golden Gate Club, San Francisco CA
2004 BMS Graduate Program Retreat, Granlibakken Tahoe City, CA
2005 BMS Student Pizza Talk, UCSF
2006 *Introduction to Research*, Department of Pathology, UCSF
2008 Division of Experimental Medicine, Divisional Seminar Series, UCSF
2009 Immunology Program, UCSF

UPCOMING INVITATIONS
2010 Breast Oncology Program Seminar, Helen Diller Family Comprehensive Cancer Center, UCSF

VII. GOVERNMENT AND OTHER PROFESSIONAL SERVICE:

GOVERNMENT SERVICE

2003-2006	National Institutes of Health, Center for Scientific Review	Ad hoc reviewer (10/2003; 02/2005; 10/2005; 06/2006), Tumor Progression & Metastasis (TPM) Study Section, Oncological Sciences Review group
2003	Division of Cancer Biology, National Cancer Institute: *Microenvironment Think Tank*	Participant and *Reporter*
2003	Division Cancer Etiology, National Cancer Institute: *Validation of A Causal Relationship: Criteria to Establish Etiology Think Tank*	Invited speaker and Participant
2004	National Institutes of Health, National Cancer Institute	Subcommittee C (05/2004) – Basic & Preclinical NCI Initial Review Group, NCI-C RPRB (T2) Angiogenesis
2005	National Institutes of Health, National Cancer Institute	Subcommittee D (02/2005) – Clinical Studies NCI Initial Review Group, NCI-D RPRB Tumor Pathology
2005	National Institutes of Health, Center for Scientific Review	Special Emphasis Panel (SEP); ZRG1 ONC (03) M, Developmental Therapeutics

OTHER PROFESSIONAL SERVICE

1999	Arkansas Science & Technology Authority	Ad hoc Grant Review
2000	McGraw-Hill, '*Biology*' 6th edition, Ed. P.H. Raven and G.B. Johnson	Ad hoc Review, Chapters 17 and 18
2000	Division of Cancer Biology, NCI: *Epithelial-Stromal Interactions & Tumor Progression Workshop*	Invited speaker and Participant
2001	Department of Veterans Affairs	Ad hoc Grant Review, Oncology Review Board
2001	Research Grants Council of Hong Kong	Ad hoc Grant Review
2003	Danish Cancer Society, DENMARK	Ad hoc Grant Review

2004	Division of Gastroenterology and Digestive Disease Research Center, Vanderbilt University, Nashville TN, USA	'*H. pylori-induced Inflammation and Gastric Adenocarcinoma*, PO1 External Advisory Panel
2004	Cancer Research Ireland, Irish Cancer Society	Ad hoc grant review
2004	Dutch Cancer Society	Ad hoc grant review
2004	Vanderbilt University, Nashville TN, USA; SPORE in GI Cancer	Ad hoc reviewer for SPORE Developmental Research Program
2005	Keystone Symposia, *Inflammation and Cancer*	Co-organizer (with Dr. Ray DuBois, Vanderbilt Univ, TN), Breckinridge, CO, USA
2006	5th Annual Timberline Symp. on Epithelial Cell Biology, '*Intrinsic and Microenvironmental Regulation of Epithelial Cancer'*	Co-Organizer (with Dr. Harold Moses, Vanderbilt University, TN, USA), Timberline, OR, USA
2006	Keystone Symposia Cancer Study Group for 2009 programming	Study group member
2007	Keystone Symposia, *Inflammation and Cancer*	Organizer (with Drs. Fran Balkwill (Cancer Research UK) and Glenn Dranoff (Beth Israel Cancer Center, Harvard, MA) Santa Fe, New Mexico, USA
2008	AACR Special Conference on '*Inflammation and Cancer'*	Co-Organizer (with Drs. Michael Karin and Larry Marnett)
2007-2010	University of Minnesota Cancer Center; Douglas Yee, M.D., Director	External Scientific Advisory Board Member
2007 – 2011	University of Washington, Seattle WA, USA	Member, External Scientific Advisory Board, CA U54 TMEN: *Significance of Microenvironment for Prostate Cancer Initiation and Progression*; P.I. Stephen R Plymate, Univ. of Washington School of Medicine.
2007 – 2011	Albert Einstein College of Medicine of Yeshiva University, New York, NY USA	Member, External Scientific Advisory Board, CA U54 TMEN: *Novel Methods for Detection Cell Interactions in the Tumor Microenvironment*; P.I. John S. Condeelis, Albert Einstein College of Medicine.
2008	International Society for Biological Therapy of Cancer (iSBTc), *2008 Workshop on Inflammation in Cancer Development*	Co-Organizer (with Drs. Michael Karin, Steven Dubinett, George Weiner)
2009	GlaxoSmith Kline	Member, Tykerb Post-ASCO KOL Advisory Board
2009	University of Southern California, Children's Hospital	Member, External Scientific Advisory Board, Neuroblastoma, Program Project grant (P01), PI: Robert Seeger, M.D.,

VIII. UNIVERSITY AND PUBLIC SERVICE

UNIVERSITY SERVICE

System wide

1992-1993	Graduate Student Representative, Dept. of Biological Chemistry Faculty Council, UCLA
2004	*ad hoc* Member External Advisory Panel; Jonsson Comprehensive Cancer Center, University of California, Los Angeles, Los Angeles CA, USA
2009	Member, Site Visit Programmatic Review Group, Department of Pathology & Laboratory Medicine, UCLA School of Medicine. Graduate Council of the UCLA Academic Senate.

University of California, San Francisco (CAMPUS-WIDE)

1997	Presentation, Donor Seminar, UCSF Development Office
1998	Presentation, Donor Seminar, UCSF Development Office
2000 – 2004	Member, Steering Committee, Ovarian Cancer Program Project Grant
2000 – 2005	Member, Scholarships and Awards Committee, Academic Senate, School of Medicine
2002 – present	Member, BioMedical Sciences Graduate Program (BMS) Executive Committee
2002 – 2004	Member, Medical Scientist Training Program Executive Committee
2004 - 2006	Member, Search Committee, Director of Molecular Imaging, Dept. of Radiology, Committee Chair: Ron Arenson, M.D. no successful recruitment
2004 – 2006	Member, BioMedical Sciences Graduate Program (BMS); Admissions Committee
2004	Organizer, BioMedical Sciences Graduate Program Retreat, Granlibakken, N. Lake Tahoe, CA USA
2005 - 2006	Member, Tissue Engineering Ladder-rank Faculty Search Committee, Dept. of Surgery, Committee Chair: Nancy Boudreau, Ph.D. Successful recruitment of Valerie Weaver, Ph.D.
2005 - 2009	Member, Ethel and Jane Sokolow Memorial Cancer Endowment Lectureship Committee.
2006	Member, Cancer Faculty Search Committee, Anatomy Dept., Committee Chair: Zena Werb, Ph.D. Successful recruitment of Jeroen Roose, Ph.D.
2006	Member, Faculty Advisory Committee for 2007 Journalist Seminar on *Inflammation and Disease*. Sponsored by Associate Vice Chancellor Barbara J. French
2007	Member, committee to select recipient of Dean's Postdoctoral Prize Lecture.
2007	Member, Faculty Search Committee for Restorative Neurosurgery and Stem Cell Neurobiology, VA Medical Center/UCSF NeuroSurgery. Committee Chair: Linda Noble, Ph.D.; Status: open.
2009	Member, Committee to choose 1st Bonnie J. and Anthony Addario Endowed Chair in Thoracic Oncology, School of Medicine, UCSF

University of California, San Francisco, Comprehensive Cancer Center

1999	Member, Cancer Center Research Building Space Review Policy Committee
1999 – 2002	Member, Mt Zion Animal Barrier Facility Committee
1999 – 2005	Member, Cancer Center Friday Seminar Series Committee
2000	Organizer and Chair, MZ Cancer Center Research Building Annual Retreat
2001	Member, 'Star Performance Award' selection committee
2001	Presentation, Evelyn Herman Reception, UCSF Development Office
2001 – 2002	Member, Cancer Center Research Building, 'Cancer Center Faculty Working Group'
2001 – 2006	Member, Mouse Models of Human Cancer Working Group
2002 – 2003	Member, UCSF Mt Zion campus, Animal Protocol Review Committee
2002	Member, ACS IRG grant review committee
2002 – 2006	Steering Committee Member, Mouse Models of Human Cancer
2003	Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award
2003 – 2009	Chair, UCSF Mt Zion Campus Animal Protocol Review Committee

2003 Member, Search Committee: Associate Director for Administration, UCSF Comprehensive Cancer Center (Erica Weber, recruited)
2004 Member, Review Committee, UCSF Comprehensive Cancer Center Stewart Trust Award
2006 Organizer, UCSF CCC Annual Symposium, *'Inflammation & Cancer: Bench to Bedside'*.
2008 Chair, Committee to nominate Postdoctoral scholar for AACR 2008 Annual Meeting, Inaugural "Future Leaders, New Directions" Special Symposium. Nominee: Laura Soucek, Ph.D. (awarded)

University of California, San Francisco, Cancer Research Institute
2001 – 2002 Member, Cancer Research Institute Membership Subcommittee

University of California, San Francisco, Department of Pathology
2003 Member, Committee to recommend faculty for the *Robert E. Smith Endowed Chair in Experimental Pathology*
2004 Member, Search Committee, Ladder rank faculty, Physician-Scientist, Anatomic Pathology. Successful recruitment of Jay Debnath, M.D., Ph.D.
2007 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Pathology and Neuropathology. Committee Chair: Michael D Prados, M.D.; Status: open.
2008 Member, Search Committee, Ladder-rank faculty, Physician-Scientist, Experimental Pathology. Committee Chair: Benedict Yen, M.D.; Status: open
2009-present Pathology Dept. Academic Merit and Promotions Committee

University (other)
2002 Guest Instructor, Graduate Oncology, University of Missouri, Columbia, Missouri USA
2003 Guest Instructor, Cancer Biology, Stanford University, Stanford, CA USA
2004 Guest Instructor, Immunology, Stanford University, Stanford, CA USA
2008 Guest Instructor, *Exploring the Tumor microenvironment*, Postgraduate course, ISREC, Lausanne University's Biochemistry and Biology Departments, and the Lausanne Branch of the Ludwig Institute, Lausanne Switzerland. Course Organizers, Ivan Stamenkovic and Michel Aguet

PUBLIC SERVICE:
1990 Lecturer, Science Academy Of Whittier, Summer Institute. Whittier College, Whittier, CA
1991 Organizer and Lecturer, Science Academy Of Whittier, Summer Institute. Whittier College, Whittier, CA.
1993 Lecturer, Joslyn Community Center. Claremont, CA.
1994 Provided elementary educators with science-related supplies (photos, slides, fixed tissue samples).
1995 Co-Coordinator Hormone Research Institute, 'Take Our Daughters To Work Day', Univ. of Calif., San Francisco
2002 Photo credits and interviewed for '*Misdiagnosis: Failure of Promising Cancer Treatment Starts Soul Searching by Researchers & Drug Companies'*, in: *San Francisco Chronicle*, May 12, 2002.
2003 Interviewed for article '*Body's First Defense May Be Root of Diseases'*, in: *The Washington Post*, February 20, 2003
2003 Interviewed for article '*The Body on Fire*', in: U.S. News & World Report, October 20, 2003
2004 Interviewed for comments in: *Science News*, '*Early Warming: Inflammatory protein tied to colon cancer risk*" February 7, 2004, Vol 165.
2004 Interviewed for article '*The Fires Within'*, in: *TIME* Magazine, February 23, 2004

2004 Interviewed for comments on AACR Annual Meeting in: *Oncology Times*, '*Exercise Reduces Inflammatory Response, May also Reduce Cancer Risk*', Robert H Carlson, 26(11):33-34, June10, 2004

2004 Interviewed for article *'Inflammation and Cancer: The Link Grows Stronger'*, in: *Science*, 306, 966-968 (2004)

2005 Interviewed for article *'Quieting a Body's Defenses'*, in: *Newsweek*, Special Edition, Summer 2005

2006 Interviewed for "Expert Commentary" by *BreastLink.org*, on article "Association Between Circulating White Blood Cell Count and Cancer Mortality." *Archives of Internal Medicine*, January 23, 2006; 166:188-194. http://www.breastlink.org/index.php?module=announce&ANN_user_op=view&ANN_id=208

2007 UCSF Research Perspectives 2007 – Inflammation as Cause and Consequences of Disease, Media Event for Journalists, September 27, 2007, UCSF Mission Bay Campus

2007 On-Air radio interview by Dave Iversen, KQED *FORUM*, September 28, 2007 San Francisco CA USA

IX. TEACHING AND MENTORING

Formal Scheduled Classes for UCSF Students:

Qtr	Academic Yr	Course No. & Title	Teaching Contribution	Units	Class Size
W	1997/98	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology*; Laboratory lecture & instruction	10	150
W	1998/99	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology;* Laboratory lecture & instruction	10	150
W	1999/00	IDS 100; Histology Laboratory	*Neoplastic Skin Histopathology*; Laboratory lecture & instruction	10	150
S	1999/00	BMS 297A; Molecular Biology & Pathology of Neoplasia	*Animal Models of Cancer Laboratory*; Laboratory lecture & instruction	3	15
S	2000/01	BMS 297A; Molecular Biology & Pathology of Neoplasia	*Animal Models of Cancer Laboratory*; Laboratory lecture & instruction	3	15
W	2000/01	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
S	2000/01	BMS 260; Cell Biology	Discussion group leader	1	6
F/W	2001/02	IDS 101; Prologue	Laboratory Instructor	9	30
W	2001/02	BMS 225; Tissue and Organ Biology	Lecture and laboratory instruction	3	15
W	2001/02	IDS 103; Cancer Block	*Invasion & Metastasis*; Lecturer	7	150
S	2001/02	BMS 260; Cell Biology	Discussion group leader	1	7
F	2002/03	BMS 260; Cell Biology	Discussion group leader	1	6
W	2002/03	IDS 103; Cancer Block	*Invasion & Metastasis*; Lecturer	7	150
F/W	2002/03	IDS 101; Prologue	Laboratory Instructor	9	30
F	2003/04	BMS 260; Cell Biology	Discussion group leader	1	6
S	2003/04	BMS 225B, Tissue and Organ Biology	Lecturer and Laboratory Instructor	1.5 - 5	tbd
W	2003/04	Biochem 297; Molecular Biology & Pathology of Neoplasia	*Angiogenesis:* Lecturer	3	30
W	2003/04	BMS 297A Molecular Biology & Pathology of Neoplasia Laboratory	Lecturer and Laboratory Instructor, *Animal Models of Neoplasia*	1	10

S	2003/04	BMS 225B; Tissue & Organ Biology	Lecturer: Cancer I & Cancer II	1.5 - 5	16
F	2004/05	BMS 260; Cell Biology	Discussion group leader	1	6
F	2005/06	BMS 260; Cell Biology	Discussion group leader	1	7
W	2006/07	Biochem 297; Molecular Biology & Pathology of Neoplasia	*Inflammation and Cancer*: Lecturer	3	30
W	2008/09	BMS230; Cellular & Molecular Biology of Cancer	Course Co-Director	3.5	22
W	2008/09	BMS230; Cellular & Molecular Biology of Cancer	Lecturer: *Cancer Microenvironments; Inflammation and Cancer*	3.5	22

Postgraduate and Other Courses:

1989	M204, *Biochemistry Lab* Univ. of Calif., Los Angeles	Student Teaching Assistant for quarter long course (100 medical students)
1989	Biology 250, *Human Heredity*; Dept. of Biology Whittier College, Whittier CA	Organized and taught entire lecture-based course (30 undergraduate students)
1990	Biology 350 & 350L, *Molecular Genetics*; Dept. of Biology, Whittier College, Whittier CA	Organized and taught entire lecture and laboratory course (16 undergraduate students)
1990	M204, *Biochemistry Lab* Univ. of Calif., Los Angeles	Student Teaching Assistant for quarter long course (100 medical students)
1990	Biology 250, *Human Heredity*; Dept. of Biology Whittier College, Whittier CA	Organized and taught entire lecture-based course (30 undergraduate students)
1992	Biology 350 & 350L, *Molecular Genetics*; Dept. of Biology, Whittier College, Whittier CA	Organized and taught entire lecture and laboratory course (16 undergraduate students)
2003	Graduate *Oncology*, University of Missouri, Columbia, MS, USA	Invited Guest Lecturer: Lecture syllabus & delivered 2-hr lecture for course (15 students, combination of graduate, medical & postgraduate fellows
2003	Graduate Program in Cancer Biology, Stanford Univ., Stanford, CA USA	Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Cancer Biology Graduate program
2004	Graduate Program in Immunology, Stanford Univ., Stanford, CA USA	Invited Guest Lecturer: Delivered 1-hr lecture to graduate students in Immunology Graduate program
2005	UCSF Dermatology residents' Basic Science Seminar Series	Invited Guest Lecturer: Delivered 1-hr lecture to UCSF Dermatology Residents (11 M.D. and M.D., Ph.D. Residents)
2008	ISREC, Lausanne University's Biochemistry and Biology Departments, and the Lausanne Branch of the Ludwig Institute	Guest Instructor: *Exploring the Tumor microenvironment*, postgraduate course. (20 PhD students, 3 hours of instruction)

High School and Undergraduate Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
1998	Christopher Tinkle	Undergraduate, Univ. of Texas, Austin, TX, USA	Summer Research Training Program Supervisor	MSTP student, Rockefeller University
2000	Adam Zucker	Undergraduate, Oberlin College, Ohio USA	Supervised Summer work	unknown
2000	Ashkan Hirari	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA, USA	Supervised Summer work	unknown
2001	Jason Reuter	Undergraduate, Univ. of Calif., Berkeley, Berkeley CA USA	Supervised Summer work	Ph.D. student, Stanford University
2002	Destinee Cooper	Undergraduate, Univ. of	Summer Research	unknown

		Calif., Davis USA	Training Supervisor	
2006	Sunum Mobin	UCSF Science & Health Education Partnership: High School Intern Program	Summer Research Training Supervisor	Intern, Coussens lab
2008-2009	Julia Lam	Univ of Calif., Berkeley	Independent study (199), Mentor	UCB undergraduate

Predoctoral Students Supervised or Mentored:

Dates	Name	Program or School	Faculty Role	Current position
2000	Jin-Sae Rhee	UCSF MSTP/BMS, graduate student	Rotation Supervisor	PhD awarded 2003, M.D. awarded 2005
2000 - 2003	Jin-Sae Rhee	UCSF M.D., Ph.D.,	Ph.D. supervisor	Pediatric Resident, Children's Hospital, Washington D.C.
2000	Maria Christophorou	UCSF BMS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 2006
2001	Leslie Chu	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2005
2001	Rayna Takaki	UCSF BMS, graduate student	Rotation Supervisor	Ph.D. awarded 2006
2001 – 2002	Sophia Bruggerman	University of Nijmegan, The Netherlands	Masters Thesis Supervisor	Ph.D. student, The Netherlands Cancer Institute
2002	Lucy Lebedeva	UCSF PIBS, graduate student	Faculty coach, BMS 297	Ph.D. awarded 20057
2002	Leslie Chu	UCSF BMS, graduate student	Ph.D. Orals committee	Ph.D. awarded 2005
2002	Andre Whitkin	MSTP student, Cornell University USA	Supervised Summer work	MSTP student, Cornell University
2002	Karin deVisser	The Netherlands Cancer Institute, The Netherlands	Ph.D. Thesis Reading Committee	Postdoctoral fellow, The Netherlands Cancer Institute
2003	Cathy Collins	UCSF MSTP student	MSTP Advisor	MSTP student, UCSF
2004	Eric Tamm	University of British Columbia, Canada	Doctoral Dissertation External Examiner	Postdoctoral fellow, Genentech Inc.,
2004	Annie Hsieh	University of Södertörn, Sweden	Masters Thesis Supervisor	unknown
2005	Geoff Benton	UCSF TETRAD/PIBS, graduate student	Ph.D. Orals committee	UCSF TETRAD PhD graduate student
2006	Morgan Truitt	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student
2006	Danielle Shin	UCSF MSTP student	Rotation Supervisor	MSTP student, UCSF
2006-2008	Celeste Rivera	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	current MS student, Coussens lab UCSF
2007-present	Leslie Vasquez	SFSU/UCSF NIH Post-baccalaureate Research Experience Program (PREP) student	M.S. research advisor	current MS student, Coussens lab UCSF
2008	Ashley Martin	UCSF BMS, graduate student	Rotation Supervisor	UCSF BMS PhD graduate student
2009-present	Kay Wiebrands	Master's Student Utrecht University, the Netherlands	Masters Thesis Internship Supervisor	current MS intern, Coussens lab UCSF
2009-present	David Tawfik	Medical Student III, UCSF	1-year rotation, Dean's Quarterly Research Fellowship	current MSIII break year, Coussens lab, UCSF

Postdoctoral Fellows and Residents Directly Supervised or Mentored

Dates	Name	Position & Funding	Faculty Role	Current Position
2000 - 2001	Ernst Lengyel, M.D., Ph.D.	Post-Doc Researcher, Senior Clinical Fellow	Research Supervisor	Assoc. Adj. Prof., Dept. Gyn. & Oncology, UCSF
2000 -2002	Leon Van Kempen, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Asst Prof., Univ. of Nijmegen, Dept. of Pathology, The Netherlands
2002 – 2005	Robert Diaz, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Scientist, Roche Pharmaceuticals
2002 – 2005	Karin deVisser, Ph.D.	Post-Doc Researcher, Dutch Cancer Society Postdoctoral Fellowship	Research Supervisor	Research Scientist, The Netherlands Cancer Institute, Amsterdam, The Netherlands
2003 – 2007	Alexandra Eichten, Ph.D.	Post-Doc Researcher, Serono Foundation for the Advancement of Medical Science (2003-2005); Coussens R01	Research Supervisor	Scientist, Regeneron Corp., New York USA
2003 - 2005	Stephen Robinson, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Private sector, United Kingdom
2003 - 2004	H. Jennifer Shen, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Post-Doctoral fellow, NIH
2005 -present	David DeNardo, Ph.D.	Post-Doc Researcher; 1) NGA: 5 T32 CA09043 PI: BISHOP; *The Molecular Analysis of Tumor Viruses;* 2) American Cancer Society Postdoctoral Fellowship 2007-2010	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2005 –2007	Nor Eddine Sounni, Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Post-Doctoral fellow, Strongin Lab, The Burnhm Inst., San Diego CA USA
2006 –2007	Tingting Tan, M.D.,Ph.D.	Post-Doc Researcher; Coussens R01	Research Supervisor	Resident, Internal Medicine, Kaiser San Francisco
2006 -present	Magnus Johansson, Ph.D.	Post-Doc Researcher; Sweedish Cancer Society Postdoctoral fellowship 2006-2008	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2006- present	Nessrine Affara, Ph.D.	Post-Doc Researcher; AACR-Astellas USA Fndt in Basic cancer Research	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2007 -present	Pauline Andreu, Ph.D.	Post-Doc Researcher; Cancer Research Institute Postdoctoral Fellowship 2008-2011	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF
2008- present	Brian Ruffell, Ph.D.	Post-Doc Researcher; Dept of Defense Postdoctoal fellowship	Research Supervisor	Post-Doctoral fellow, Coussens Lab, UCSF

FACULTY MENTORING

Faculty Mentored:

Dates	Name	Position while Mentored	Mentoring Role	Current Position
2001 – 2004	Ernst Lengyel, M.D., Ph.D.	Asst. Adjunct Professor	Research Mentor	Asst. Prof., Dept. Gyn. & Oncology, Univ. of Chicago, Chicago, IL

2002 – 2007	Darya Soto, M.D.	Asst. Adjunct Professor,	K08 Research Mentor	Private Practice, Burlingame, CA
2005 – 2007	Runi Chattopadhyay, M.D.	Clinical Instructor and Clinical Fellow	Basic Science Mentor, K12	Director, Breast Center, California Pacific Med. Center, San Francisco CA
2006 – present	Limin Liu, Ph.D.	Assistant Professor	Member, Mentoring Committee	Dept. of Microbiology & Immunology, Sandler Center for Basic Research in Asthma, UCSF

Sabbatical Visitors:
1999 - 2000 Yves DeClerck, M.D. Professor, Univ. of Southern Calif. & Children's Hospital of Los Angeles

SUMMARY OF TEACHING HOURS

Academic Year	Teaching/Mentoring Summary	Hours
1997/98	Total hours of teaching /mentoring:	27
	Formal class or course teaching hours:	2
	Informal teaching hours including prep time:	1
	Mentoring hours:	24
1998/99	Total hours of teaching /mentoring:	71
	Formal class or course teaching hours:	2
	Informal teaching hours including prep time:	1
	Mentoring hours:	68
1999/00	Total hours of teaching /mentoring:	108
	Formal class or course teaching hours:	4
	Informal teaching hours including prep time:	2
	Mentoring hours:	102
2000/01	Total hours of teaching /mentoring:	130
	Formal class or course teaching hours:	16
	Informal teaching hours including prep time:	9
	Mentoring hours:	105
2001/02	Total hours of teaching /mentoring:	201
	Formal class or course teaching hours:	18
	Informal teaching hours including prep time:	19
	Mentoring hours:	164
2002/03	Total hours of teaching /mentoring:	314.5
	Formal class or course teaching hours:	15.5
	Informal teaching hours including prep time:	17
	Mentoring hours:	282
2003/04	Total hours of teaching /mentoring:	402
	Formal class or course teaching hours:	20
	Informal teaching hours including prep time:	28
	Mentoring hours:	354
2004/05	Total hours of teaching /mentoring:	395
	Formal class or course teaching hours:	17
	Informal teaching hours including prep time:	28
	Mentoring hours:	350

2005/06	Total hours of teaching /mentoring:	395
	Formal class or course teaching hours:	17
	Informal teaching hours including prep time:	28
	Mentoring hours:	350
2006/2007	Total hours of teaching /mentoring:	473
	Formal class or course teaching hours:	45
	Informal teaching hours including prep time:	28
	Mentoring hours	400
2008/2009	Total hours of teaching /mentoring:	499
	Formal class or course teaching hours:	51
	Informal teaching hours including prep time:	48
	Mentoring hours	400

X. RESEARCH AND CREATIVE ACTIVITIES

RESEARCH AWARDS AND GRANTS:

CURRENT

P30 CA82103 (PI: McCormick, F; UCSF) 8/5/1999-5/31/2012
Source: NIH/NCI $5,058,311 (directs)
Title: *Cancer Center Support Grant*
Role: Co-Director, Mouse Pathology Shared Resource
The Cancer Center Support Grant provides support for administration and infrastructure for the UCSF Comprehensive Cancer Center. Dr. Coussens is the Core Co-Director of the Mouse Pathology Shared Resource that provides routine hematologic and histopathologic processing of tissue and blood samples to members of the UCSF community.

BC051640 Era of Hope Scholar Award (PI: Coussens, LM; UCSF) 06/01/06 – 05/31/11
Source: Department of Defense Breast Cancer Research Program (BCRP) of the $454,111 directs yr 1
the Congressionally Directed Medical Research Programs (CDMRP**)** $2,347,728 total directs
Title: Microenvironment Regulation of Mammary Carcinogenesis
Role: PI
The goal of this Scholar Award is to identify leukocytes and their proteases that modify breast carcinogenesis and to develop noninvasive imaging reagents targeting leukocytes to image inflammation.

RO1 CA132566 (multi PI: Coussens, LM; Jablons DM; UCSF) 05/01/08-04/30/13
Source: NIH/NCI $190,000 directs/yr
Title: Inflammation and Lung Carcinogenesis $1,467,591 directs/yr 1-5
Role: Principal Investigator
The goal of this study is to determine how inflammation and Wnt signaling regulate stem cell niche autonomy during lung carcinogenesis

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RO1 CA130980 (PI: Coussens, LM; UCSF) 07/01/08-06/30/13
Source: NIH/NCI $207,500 directs/yr
Title: Regulation of Inflammation-Associated Epithelial Cancer Development
Role: Principal Investigator
The goal of this study is to determine regulatory programs activating chronic inflammation during squamous carcinogenesis

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W81XWH-08-PRMRP-IIRA (multi-PI: Broaddus, C; Coussens, LM; UCSF) 07/01/09 – 06/30/12
Source: DOD $289,840 directs/yr
Title: Role of Macrophage-induced Inflammation in Mesothelioma
The goals of this project are 1) to determine the functional significance of macrophage phenotype in mesothelioma, 2) to determine the functional significance of macrophages as regulators of mesothelioma apoptosis in vitro and 3) to define the functional significance of macrophage depletion or repolarization on mesothelioma survival in vivo.
Role: P.I.

P50 CA58207 (Gray; LBNL/UCSF) 08/01/92 – 11/30/12
Source: NIH/NCI $40,00 (project expenses only)
1/1/09-12/31/09
Title: Bay Area Breast Cancer SPORE
Career Development and Developmental Research Award, Multi Project PI: Boudreau N; Coussens LM
Title: Macrophage-Mediated Delivery of the Breast Tumor Suppressor HoxD10 via Autologous Transfer to Breast Tumors. The aims of this project are to 1) establish function and optimize introduction of the engineered HoxD10 protein into macrophages and/or monocytes; 2) visualization of modified monocyte/macrophage accumulation in mammary tumors in vivo and 3) analysis of the impact of monocyte/macrophage delivered HoxD10 on breast tumor growth, progression and metastasis in MMTV-PyMT mouse model of mammary carcinogenesis.
Role: Multi P.I.

R01CA140943 (multi-PI: Coussens, Boudreau, Daldrup-Link; UCSF) 07/01/09 – 06/30/13
Source: NIH/NCI $200,000 directs/yr
Title: Improved Imaging and Drug Delivery Using Novel Approaches to Regulate Tissue Perfusion
The major goal of this project is to examine how short-term inhibition of ALK5 in vivo alters hemodynamics and tissue perfusion in mouse models of cancer.
Role: P.I.

PREVIOUS

USPHS 5 T32 CA09056 (PI: Fox, F, UCLA) 07/01/89 –06/30/92
Source: NIH/UCLA $9,300 directs/yr1
Title: Regulation of *junB* Gene Expression by TGF-Beta $25,800 directs/yr 1-3
Competitive Pre-Doctoral award to study transcription factor *junB.*

Univ. of Calif., Dissertation Year Fellowship (PI: Coussens, LM, UCLA) 10/1/92 – 09/31/93
Source: University of California, Office of the President $13,350 directs/yr
Title: *Effects of E1A on TGF-Beta-inducible junB Expression*
Competitive Pre-Doctoral award to study transcription factor *junB.*

USPHS 5 T32 CA09043 (PI: Bishop, KM, UCSF) 10/01/93-06/31/96
Source: NIH/UCSF $25,000 directs/yr
Title: *Molecular Analysis of Tumor Viruses* $75,000 directs/yr 1-3

Post-Doctoral fellowship to study mouse model of epithelial carcinogenesis.

American Social Health Association/Pfizer Post-Doctoral Research Fellowship in Sexually Transmitted Diseases (PI: Coussens, LM, UCSF) 10/01/96 – 9/30/98
Source: Private Foundation $27,500 directs/ yr 1
Title: *Metalloproteinases and Malignant Progression of Squamous Epithelium in K14-HPV16 Transgenic Mice* $56,250 directs/yr 1-2
Role: Principal Investigator
Competitive Post-Doctoral fellowship to study proteases and tumor development .

P01 CA072006 (PI: Shuman M, UCSF) 06/10/97 – 06/30/03
Source: NIH/NCI $803,021 directs/yr 1
Title: *Proteases in Cancer Biology and Drug Development* $4,280,649 directs/yr 1-5
Project 3 – Proteases in Models of Tumor Initiation/Progression $165,438 directs/yr 1
Role: Co-Investigator, Project 3 $940,281 directs/yr 1-5
The major goal of this project is to study the role of proteases in cancer biology. $72,595 directs/yr 1
Core C – Transgenic Animal Models $470,620 directs/yr 1-5
Role: Director (year 4 and 5)
The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

UCSF IRG-97-150-01 (PI: Coussens LM, UCSF) 07/01/99-06/30/00
Source: American Cancer Society $20,000 directs/yr 1
Title*: Proteases and Genomics in a Mouse Model of Epithelial Cancer* $20,000 directs/yr 1
Role: Principal Investigator
Pilot project tested role of proteinases as effectors of genomic instability.

UCSF Cell Cycle and Dysregulation Program (PI: Coussens LM, UCSF) 02/01/00-01/31/01
Source: UCSF Comprehensive Cancer Center, Intramural $14,000 directs/yr 1
Title*: Epithelial Neoplastic Progression and Degradation of Type ICollagen* $14,000 directs/yr 1
Role: Principal Investigator
Pilot project assessed functional significance of type I collagen metabolism during epithelial carcinogenesis.

Research Evaluation and Allocation Committee (PI: Coussens LM, UCSF) 07/01/00-06/30/01
Source: UCSF Academic Senate $30,000 directs/yr 1
Title: *Role of Gelatinase B in Maintenance of Genomic Instability* $30,000 directs/yr 1
Role: Principal Investigator
Pilot project tested the role of MMP9 as an indirect regulator of genomic instability.

UCSF IRG AC-04-02 (PI: Coussens LM, UCSF) 10/1/00-09/30/01
Source: American Cancer Society $20,000 directs/yr 1
Title: *Regulation of Intracellular Signaling Pathways by Gelatinase B/MMP-9* $20,000 directs/yr 1
Role: Principal Investigator
Pilot project to study signal transduction pathways regulated by MMP-9.

The V Foundation for Cancer Research (PI: Coussens LM, UCSF) 06/02/00-05/31/02
Source: Private Foundation $50,000 directs/yr 1

Title: *Gelatinase B and Epithelial Cancer Development* $100,000 directs/yrs 1-2
Role: Principal Investigator
Pilot project to study role of MMP9 during epithelial carcinogenesis.

Gertrude B. Elion Cancer Research Award (PI: Coussens LM, UCSF) 07/1/01 – 06/30/02
Source: American Association of Cancer Research $50,000 directs/yr 1
Title: *Functional Role of MMP-2 During Epithelial Carcinogenesis* $50,000 directs/yr 1
Role: Principal Investigator
Pilot project to study role of MMP-2 during epithelial carcinogenesis.

Univ. of Calif., Cancer Research Coordinating Committee (PI: Coussens LM, UCSF) 07/1/01 – 06/30/02
Source: University of California $48,874 directs/yr 1
Title: *Gelatinase A/MMP-2 and Epithelial Cancer Development* $48,874 directs/ yr 1
Role: Principal Investigator
Pilot project to study role of MMP-2 as a potentiator of tumor development.

Hellman Family Award For Early Career Faculty (PI: Coussens LM, UCSF) 11/1/00-09/30/02
Source: UCSF Intramural $49,000 directs/ yr 1
Title: *Paracrine Regulation of Epithelial Carcinogenesis by MMP-9* $89,000 directs/yr 1-2
Role: Principal Investigator
Pilot project to identify matrix molecules regulated by MMP-9.

Edward Mallinckrodt, Jr. Foundation (PI: Coussens LM, UCSF) 10/1/00-09/30/03
Source: Private Foundation $61,000 directs/yr 1
Title: *Regulation of epithelial cancer by gelatinase B/MMP-9* $194,000 directs/yr 1-3
Role: Principal Investigator
Pilot project to determine how MMP-9 regulates proliferation, VEGF bioavailability and angiogenesis during epithelial carcinogenesis.

P50 CA58207 (PI: Gray, J: UCSF) 03/01/03-02/28/05
Source: NIH/NCI $50,000 directs/yr 1
Bay Area Breast Cancer Translational Research Program (SPORE) $100,000 directs/yr 1-2
Title: *Type I Collagen Remodeling and Mammary Carcinogenesis*
Role: Principal Investigator (Developmental Project)
The overall goal of this pilot project was to explore the role of collagen metabolism during mammary carcinogenesis.

DE-FG02-05ER6401 (PI: Franc, B**;** UCSF**)** 03/01/05 – 01/16/06
Source: DOE Medical Applications Grant $225,100 directs yr 1
Title: Therapeutic Radionuclide Tumor-targeting Strategy for Breast Cancer $1,125,500 total directs
Role: Co-Investigator
The specific aim of this project is to develop a radionuclide delivery molecule (RDM) that specifically targets cancer cells that express matrix-metalloproteinase-14 (MMP-14) on their surface and demonstrate delivery of radiolabeled RDM to MMP-14 expressing cells *in vitro* and *in vivo.*

R01 DK067678 (PI: Cher, M: Wayne State University) 7/1/03-6/30/06
Source: NIH/NIDDK $14,675 directs/yr 1
Title: *Proteases in Prostate Cancer Bone Metastasis* $122,794 directs/yr 1-4

Role: Subcontract Principal Investigator
The major goal of this subcontract is to assist with the planned experiments by providing mice (protease deficient) of defined genotype for proposed studies to analyze proteases during prostate metastasis to bone *in vivo*.

Opportunity Award, Sandler Family (PI: Coussens, LM; UCSF) 02/15/05 -02/14/07
Source: UCSF Intramural $95,000 directs yr 1
Title: *B Lymphocytes as Targets for Cancer Prevention* $191,000 total directs
Role: Principal Investigator
The major goal of this project is to investigate the efficacy of targeting B cells for chemoprevention

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DAMD17-02-1-0693 (PI: Sloane, B; Wayne State University) 08/01/02-07/31/06
Source: Department of Defense $5,746,832 directs/yr 1-4
Breast Cancer Center of Excellence $49,576 directs/yr 1
Title: *Validation of Proteases as Therapeutic Targets in Breast Cancer Functional Imaging of Protease Expression, Activity and Inhibition*
Role: Subcontract Principal Investigator $198,307 directs/yr 1-4
The goal of this program is to validate proteases as therapeutic targets in breast cancer by functional imaging of protease expression, activity and inhibition.

R01 CA94168 (PI: Coussens, LM: UCSF) 04/1/02-06/31/07
Source: NIH/NCI $222,500 directs/yr 1
Title: *Regulation of Epithelial Cancer by MMP-9/gelatinase B* $1,112,500 directs/yr 1-5
Role: Principal Investigator
The goal of this project is to identify molecules that mediate proliferative and cellular pathways activated by MMP-9.

U54 RR020843 (PI: Smith, J; Burnham Institute) 09/30/04-07/31/06
Source: NIH/National Center for Research Resources $1,916,878 directs/yr 1-5
Title: Center on Proteolytic Pathways
Role: Principal Investigator (Driving Biological Problem #1) $67,306 directs/yr
DBP#1 *Proteolytic Pathways in Acute Vascular Response*

P01 CA72006 (PI: Werb, Z; UCSF) 07/07/03 – 06/30/08
Source: NIH/NCI $1,523,691 directs/yr 6
Title: *Proteases in Cancer Biology and Drug Development* $6,354,685 directs/yr 6-11
Project 3 - *Proteases in Models of Tumor Initiation/Progression* $229,788 directs/yr 6
Role: Co-Investigator, Project 3 $1,172,879 directs/yr 6-11
The major goal of this project is to study the role of proteases in cancer biology.
Core C - *Transgenic Animal Models* $151,612 directs/yr 6
Role: Director $765,974 directs/yr 6-11
The major goal of this Core is to develop and provide protease null and transgenic mice to program projects.

R01 CA98075 (PI: Coussens, LM; UCSF) 07/1/03-06/30/09 (1-yr no cost extension)
Source: NIH/NCI $222,500 directs/yr 1
Title: *Microenvironmental Regulation of Tumor Progression* $1,112,500 directs/yr 1-5

Role: Principal Investigator
The overall goal of this grant is to determine the role of collagen metabolism on epithelial carcinogenesis.

XI. PEER REVIEWED PUBLICATIONS

1. Francke U, de Martinville B, **Coussens L**, Ullrich A. (1983) The human gene for the Beta subunit of nerve growth factor is located on the proximal short arm of chromosome 1. *Science* 222:1248-1251.
2. Breakefield X, Castiglione C, **Coussens L**, Axelrod F, Ullrich A. (1984) Structural gene for Beta-nerve growth factor is not defective in familial dysautonomia. *Proc. Natl. Acad. Sci. USA* 81:4213-4216.
3. Ullrich A, **Coussens L**, Hayflick J, Dull T, Gray A, Tam A, Lee J, Yarden Y, Libermann T, Schlessinger J, Downward J, Bye J, Whittle N, Waterfield M, Seeburg P. (1984) Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309:418-425.
4. Ullrich A, Bell J, Chen E, Herrera R, Petruzzelli L, Dull T, Gray A, **Coussens L**, Liao Y-C, Tsubokawa M, Mason A, Seeburg P, Grunfield C, Rosen O, Ramachandran J. (1985) Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* 313:756-761.
5. Lauffer L, Garcia P, Harkins R, **Coussens L**, Ullrich A, Walter P. (1985) Topology of the signal recognition particle receptor in the endoplasmic reticulum membrane. *Nature* 318:334-338.
6. Schechter A, Hung M-C, Vaidanathan L, Weinberg R, Yang-Feng T, Francke U, Ullrich A, **Coussens L.** (1985) The *neu* gene: An *erbB*-homologous gene distinct from and unlinked to the gene encoding the EGF receptor. *Science* 229:976-978.
7. **Coussens L**, Yang-Feng T, Liao T-C, Chen E, Gray A, McGrath J, Seeburg P, Libermann T, Schlessinger J, Francke U, Levinson A, Ullrich A. (1985) Tyrosine kinase receptor with extensive homology to the EGF receptor shares chromosomal location with *neu* oncogene. *Science* 230:1132-1139.
8. **Coussens L**, Van Beveren C, Smith D, Chen E, Mitchell R, Isacke C, Verma I, Ullrich A. (1986) Structural alteration of viral homologue of receptor proto-oncogene *fms* at carboxyl terminus. *Nature* 320:277-280.
9. Parker P, **Coussens L**, Totty N, Rhee L, Young S, Chen E, Stabel S, Waterfield M, Ullrich A. (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* 233:853-859.
10. **Coussens L**, Parker P, Rhee L, Yang-Feng T, Chen E, Waterfield M, Francke U, Ullrich A. (1986) Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. *Science* 233:859-866.
11. **Coussens L**, Rhee L, Parker P, Ullrich A. (1987) Alternative splicing increases the diversity of the human protein kinase C family. *DNA* 6:389-394.
12. Yarden Y, Kuang W-J, Yang-Feng T, **Coussens L**, Munemitsu S, Dull T, Schlessinger J, Francke U, Ullrich A. (1987) Human proto-oncogene *c-kit*: A new cell surface receptor-tyrosine kinase for an unidentified ligand. *EMBO J*. 6:3341-3351.
13. MacDonald R, Pfeffer S, **Coussens L**, Tepper M, Brocklebank C, Mole J, Anderson J, Chen E, Czech M, Ullrich A. (1988) A single receptor binds both insulin-like growth factor II and mannose-6-phosphate. *Science* 239:1134-1137.
14. Formby B, Ullrich A, **Coussens L**, Walker L, Peterson C. (1988) Growth hormone stimulates insulin gene expression in cultured human fetal pancreatic islets. *J. Clin. Endo. Metab.* 66:1075-1079.
15. Mosthaf L, Grako D, Dull T, **Coussens L**, Ullrich A, McClain D. (1990) Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO J.* 9:2409-2413.
16. **Coussens LM**, Yokoyama K, Chiu R. (1994) Transforming Growth Factor β_1-mediated induction of *junB* is selectively inhibited by expression of Ad.12-E1A. *J. Cell. Physio.* 160:435-444.

17. **Coussens LM**, Hanahan D, Arbeit J. (1996) Genetic predisposition and parameters of malignant progression in K14-HPV16 transgenic mice. *Am. J. Pathol.* 149:1899-1917.
18. **Coussens LM**, Werb Z. (1996) Matrix metalloproteinases and the development of cancer. *Chemistry and Biology* 3:895-904.
19. Bergers G, Hanahan D, **Coussens LM.** (1998) Angiogenesis and apoptosis are cellular parameters regulating neoplastic progression in transgenic mouse models of tumorigenesis. *Int. J. Dev. Biol. 42*:995-1002.
20. **Coussens LM,** Raymond WW, Bergers G, Laig-Webster M, Behrendtsen O, Werb Z, Caughey, GH, Hanahan D. (1999) Inflammatory mast cells upregulate angiogenesis during squamous epithelial carcinogenesis. *Genes & Development* 13:1382-1397.
21. McKerrow JM, Bhargava V, Hansell E, Kuwahara T, Matley M, **Coussens LM,** Warren R. (2000) A functional proteomics screen of proteases in colorectal carcinomas. *Molecular Medicine* 6:450-460.
22. Bergers G, **Coussens LM.** (2000) Extrinsic regulators of epithelial tumor progression: metalloproteinases. *Curr. Opin. Genetics & Development* 10:120-127.
23. **Coussens LM,** Tinkle CL, Hanahan DH, Werb Z. (2000) MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 103:481-490.
24. van Kempen LCL, Rhee JS, Dehne K, Lee J, Edwards DR, **Coussens LM**. (2002) Epithelial carcinogenesis: Dynamic interplay between neoplastic cells and their microenvironment. *Differentiation* 70: 610-623.
25. **Coussens LM**, Fingleton B, Matrisian LM. (2002) Matrix metalloproteinases and cancer: Trials and tribulations. *Science* 295:2387-2392.
26. **Coussens LM**, Werb Z. (2002) Inflammation and cancer. *Nature* 420: 860-867.
27. Daniel D, Meyer-Morse N, Bergsland EK, Dehne K, **Coussens LM,** Hanahan D. (2003) Immune enhancement of skin carcinogenesis by $CD4^+$ T cells. *J Exp. Med*, 197:1017-1028.
28. van Kempan LCL, Ruitter DJ, van Muijen GNP, **Coussens LM.** (2003) The tumor microenvironment: a critical determinant of neoplastic evolution. *Euro J Cell Biol*, 82:539-548.
29. Rhee JS, Diaz R, Korets L, Hogson G, **Coussens LM**. (2004) TIMP-1 alters susceptibility to carcinogenesis. *Cancer Research* 64:952-961.
30. Chantrain CF, Shimada H, Groshen S, Ye W, Shalinsky DR, Werb Z, **Coussens LM**, DeClerck YA. (2004) Stromal matrix metalloproteinase-9 (MMP-9) regulates the vascular architecture in neuroblastoma by promoting pericyte recruitment. *Cancer Research* 64:1675-1686.
31. Baluk P, Raymond WW, Ator E, **Coussens LM**, McDonald DM, Caughey GH. (2004) Matrix metalloproteinase-2 and -9 expression increases in mycoplasma-infected airways but is not required for vascular remodeling. *Am J Physiol Lung Cell Mol Physiol*, 287:307-317.
32. de Visser KE, Korets LV, **Coussens LM**. (2004) Early neoplastic progression is complement-independent. *Neoplasia* 6: 768-776.
33. Jodele S, Chantrain CF, Blavier L, Crooks GM, Shimada H, **Coussens LM**, DeClerck YA. (2005) The contribution of bone marrow-derived cells to the tumor vasculature in neuroblastoma is matrix metalloproteinase-9-dependent. *Cancer Research*, 65: 3200-3208.
34. de Visser KE, Korets LV, **Coussens LM**. (2005) De novo carcinogenesis promoted by chronic inflammation is B lymphocyte dependent. *Cancer Cell* 7:411-423.
35. de Visser KE, **Coussens LM**. (2005) The interplay between innate and adaptive immunity regulates cancer development. *Cancer Immunology & Immunotherapy*, 54:1143-1152.
36. de Visser KE, Eichten A, **Coussens LM**. (2006) Paradoxical roles of the immune system during cancer development. *Nature Reviews Cancer*, 6:24-37.
37. van Kempen LCL, de Visser KE, **Coussens LM**. (2006) Inflammation, Proteases and Cancer. *Euro J Cancer*, 42: 728-734.

38. Yuan F, Verhelst HL, Blum G, **Coussens LM**, Bogyo M. (2006) A selective activity-based probe for the papain family cysteine protease dipeptidyl peptidase I/Cathepsin C. *J Am Chem Society*, 128: 5616-5617.
39. Tan TT, **Coussens LM**. Humoral immunity, inflammation and cancer. (2007) *Curr Opin Immunology* 19(2), 209-216
40. Johansson M, Tan T, de Visser KE, **Coussens LM**. (2007) Immune cells as anti-cancer therapeutic targets and tools. *J Cellular Biochemistry*, 101: 918-926.
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XII. PATENTS

1. U.S. Patent Application Serial No. 10/567,873
   Title: *Novel Indications for Transforming Growth Factor-Beta Regulators*.
   Inventors: **Lisa M. Coussens** and Zena Werb
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XIV. RESEARCH PROGRAM:

The overall goal of my research program is to define cellular, molecular and microenvironmental determinants of cancer development. My research is based upon the premise that, in addition to intrinsic changes occurring within neoplastic cells, e.g., activation of oncogenes and inactivation of tumor suppressor genes, extrinsic factors, e.g., inflammation, extracellular matrix (ECM) remodeling and angiogenesis also regulate critical properties of tumor evolution.

During the early development of cancer, many physiological processes occur in the vicinity of 'young tumor cells' that are similar to processes that occur during embryonic development and to healing of wounds in adult tissue, e.g., leukocyte infiltration, activation of pre-existing vasculature and development of new blood supply (angiogenesis) and extensive tissue remodeling. During wound healing, immune cells are recruited to sites of injury to eliminate potential bacterial infection as well as to facilitate healing by providing growth factors and proteases that are essential to the process. In so doing, tissue remodeling occurs and accommodates generation of a new blood supply that further helps the tissue heal. When 'healing' is complete, inflammation and tissue remodeling resolves and the tissue returns to its former state. Several of these parameters are conserved during tumor development; however, instead of initiating a 'healing' response, inflammatory cells, tissue remodeling and angiogenesis provides growth-promoting factors that promote tumor development. By studying mouse models of skin, breast and lung cancer development, the Coussens lab is identifying important cellular pathways and molecules involved in regulating tumor-associated inflammation, tissue remodeling and angiogenesis. Identification of these important regulatory mechanisms will reveal drug-targets that can then be used to design novel therapeutic strategies for treating and imaging cancer development in humans.

I. INFLAMMATION AND CANCER

The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated[1,2]. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. To address these issues, we have taken a several approaches to investigate mechanisms involved in: *i.* induction and maintenance of inflammatory microenvironments in premalignant tissues, *ii.* role of leukocyte proteases as regulators of cancer development, and *iii.* development of novel non-invasive imaging reagents to monitor and/or delivery radiotherapeutics to carcinoma cells. Our studies are designed to test the hypothesis that *inflammation* is a critical parameter of neoplastic development and therefore represents an efficacious target for anti-cancer therapies.

***THE INTERPLAY BETWEEN ADAPTIVE AND INNATE IMMUNITY PROMOTES CANCER DEVELOPMENT*:** Recent clinical and experimental data suggest a strong promoting role for inflammatory cells during early neoplastic development. One aim of our study is to understand the mechanisms regulating recruitment of infiltrating leukocytes towards premalignant epithelial lesions and to understand how they functionally contribute to tumor evolution. For these studies, we have taken advantage of a transgenic mouse model of squamous cell carcinoma (SCC) development where human papillomavirus type 16 (HPV16) oncogenes are expressed in mitotically active basal keratinocytes in skin epidermis, e.g., K14-HPV16 transgenic mice[3]. By genetically modulating the host immune response and/or presence of various leukocyte-specific extracellular proteinases with gene knockouts and double transgenic mice, we have been able to manipulate the temporal dynamics, overall incidence and malignant potential of carcinomas that develop in HPV16 mice[3-8]. Recently we demonstrated that B lymphocytes were required for initiating chronic infiltration of innate immune cells into premalignant tissue and promotion of de novo epithelial carcinogenesis in HPV16 mice[8]. Adaptive immune-deficiency in HPV16 mice results in failure to recruit innate immune cells into premalignant tissue, and as a consequence, responding pathways downstream of inflammatory cell activation and infiltration, e.g. tissue remodeling, angiogenesis, keratinocyte hyperproliferation and cancer development, are significantly attenuated. Importantly, the necessary characteristics of premalignant progression were restored by transfer of B lymphocytes or serum from HPV16 mice into T and B lymphocyte-deficient/HPV16 mice, indicating that B lymphocytes, from a distal locale, play a crucial role as initiators of chronic inflammation associated with premalignant progression, and thus potentiate the neoplastic cascade downstream of oncogene expression. While

there have been many studies on the role of B lymphocytes and/or immunoglobulins as mediators of acute inflammation, this is the first to provide clear and convincing functional proof, via the combined use of gene knock out mice, adoptive B cell transfer and serum transfer, to demonstrate that B cells and/or humoral immunity exerts an important roles in epithelial carcinogenesis, and particularly at the earliest stages of carcinogenesis, during premalignant progression.

Since genetic elimination of $CD4^+$ and $CD8^+$ T cells[5,8]or the critical complement component C3[6] did not result in reduced leukocyte infiltration or altered characteristics of premalignancy, and since neoplastic progression in HPV16 mice is characterized by abundant deposition of IgG and IgM molecules into skin interstitial environments, we are currently testing the hypothesis that activation of B cells in secondary lymphoid organs and induction of humoral immune responses with local deposition of immunoglobulin into initiated skin play crucial roles in the recruitment pathway regulating leukocyte infiltration and potentiation of squamous carcinogenesis. The implications of these findings support a model in which inhibition of leukocyte recruitment or neutralization of leukocyte activation in premalignant tissue may significantly alter characteristics of neoplastic development.

LEUKOCYTE PROTEASES AS REGULATORS OF CANCER DEVELOPMENT: Squamous carcinogenesis in HPV16 mice is characterized by infiltration of premalignant tissue by innate immune cells, specifically mast cells (MC) and neutrophils, that occurs prior to induction of epithelial hyperproliferation, loss of keratinocyte terminal differentiation or activation of angiogenic vasculature[9,10] occurs. We have reported that specific leukocyte-derived extracellular proteases, e.g., matrix metalloproteinase-9 (MMP-9), MC chymase (mMCP4) and MC tryptase (mMCP6), promote premalignancy by initiating tissue remodeling in hyperplasias and regulate bioavailability of growth factors that enhance angiogenesis and epithelial hyperproliferation[9,10]. More recently, we identified a critical regulator of these leukocyte proteases, e.g. *dipeptidyl peptidase I* (DPPI, Cathepsin C), that also appears to exert a significant impact on neoplastic progression.

Cathepsin C is lysosomal cysteine-class hydrolase expressed in most mammalian tissues. In myeloid cells and cytotoxic lymphocytes, it is found in secretory granules where it is solely responsible for NH_2-prodipeptide removal and catalytic activation of several leukocyte-derived serine proteases, including mast cell chymases (mMCP4 and mMCP5), neutrophil elastase (NE), cathepsin G, and cytotoxic lymphocyte-derived granzymes A and B[11-14], thus implicating cathepsin C as a regulator of thrombin regulation, fibroenctin turnover[15], angiogenesis[16-18], acute experimental arthritis[14], cytotoxic lymphocyte-mediated apoptosis and host immune defense[11]. Based upon these implications, cathepsin C may represent a tractable target for therapeutic intervention in a diverse array of human disease states such as wound healing, recovery from sepsis, and diseases associated with chronic inflammation, e.g., arthritis, inflammatory bowl disorders and cancer development. To test this hypothesis and to determine how cathepsin C may contribute to cancer development, we introduced a homozygous null mutation in the *Cathepsin C* gene into HPV16 mice and found significant attenuation of leukocyte migration into premalignant skin, failure to activate angiogenic vasculature, restricted keratinocyte proliferation and reduced cancer development (manuscript in preparation). Interestingly, activation of adaptive immune responses was not compromised by cathepsin C deficiency indicating that its major role is in regulation of innate immune cell activation, response and function. Since the activity of MC chymase, MC tryptase, neutrophil elastase and MMP-9 in HPV16/cathepsin $C^{-/-}$ mice was found to be >1.0% of control HPV16 mice, we hypothesize that cathepsin C regulates cancer developement via its ability to activate a diverse array of leukocyte-specific proteases that are themselves critical for the early biologies associated with premalignancy in skin. To test this, we are currently, *i.* identifying cathepsin C substrates *in vivo*, *ii.* investigating which leukocyte proteases activated by cathepsin C are significant for regulating each of the discrete biologies attenuated in HPV16 cathepsin C-deficient mice, *iii*. developing novel activity-based probes for assessing cathepsin C activity[19] and other leukocyte proteases activities *in vivo*.

IMPACTING BREAST CANCER: It is well established that chronic inflammation contributes to epithelial cancer development[20,21]. Many studies have suggested that inflammatory leukocytes promote cancer development by providing soluble growth and survival factors to initiated cells and contribute to tissue remodeling and angiogenesis via delivery of extracellular proteases[20]. However, molecular mechanisms responsible for initiation and/or maintenance of leukocytic infiltrates associated with developing breast neoplasms and/or the downstream consequences of activated leukocytes in premalignant mammary tissue are inadequately understood. We have previously demonstrated that squamous carcinogenesis is accompanied by sustained presence of specific innate immune cells during premalignant progression. Moreover, we revealed that neutralizing the effects of chronic leukocyte infiltration by either attenuating their presence or eliminating specific extracellular proteases they express

reduces cancer development[8,9]. Together, these studies indicate that skin cancer is susceptible to immunomodulatory therapeutic interventions. **Our goal is to translate our success in skin to the mammary gland and determine the degree to which breast cancer is susceptible to immuno-modulation as a therapeutic approach.** To achieve this, we are utilizing state-of-the-art three dimensional (3D) organotypic mammary epithelial models[22] and transgenic mouse models of mammary carcinogenesis, e.g., MMTV-PyMT[23] and MMTV-Neu[24] and cutting-edge *in vivo* imaging technology to identify, validate and image key immunomodulators that regulate breast cancer development in a paracrine manner. That anti-inflammatory agents have already demonstrated efficacy as anti-breast cancer agents[25,26] and are being confirmed in ongoing cancer prevention trials supports the clinical relevance for our approach and goal.

We have examined the presence of leukocytes during human breast cancer progression by immunohistochemical detection of several leukocyte-specific molecules, e.g., CD45 (leukocyte common antigen), CD68 (macrophages), CD3 (lymphocytes), CD119 (mast cells) and GR-1 (neutrophils), and revealed that as breast epithelial cells undergo progression to malignancy, the number of intraductal leukocytes present in stroma increases in parallel with the development of breast cancer[21,27]. To study the functional significance of these diverse leukocytes and to identify mouse models in which to examine their role, we have similarly examined the profile of leukocytes in two mouse models of mammary carcinogenesis, e.g., MMTC-PyMT and MMTV-neu, and have found similar profiles of infiltrating leukocytes as compared to human clinical material.

CATHEPSIN C AS A REGULATOR OF MAMMARY CARCINOGENESIS: In collaboration with our european colleague Dr. Dylan Edwards (University of East Anglia, UK), we have examined relative mRNA expression for the lysosomal cysteine protease cathepsin C during neoplastic progression in the MMTV-PyMT mouse model of mammary carcinogenesis. Our results indicate that as mammary epithelial cells undergo neoplastic progression and develop large adenocarcinomas, the relative mRNA expression for cathepsin C increases ~7-fold. Immunohistochemical analysis of cathepsin C protein expression on tissue samples from MMTV-PyMT mice indicate increased protein presence in both mammary epithelial cells and stromal cells within large adenocarcinomas as well as in lung metastases.

To address the functional significance of cathepsin C in epithelial versus stromal inflammatory cells, we are generating chimeric MMTV-PyMT mice harboring cathepsin C-deficient myeloid cells and cathepsin C-proficient epithelium (n=20) as compared with MMTV-PyMT mice harboring cathepsin C-proficient myeloid cells and cathepsin C-deficient epithelium (n=20). Chimeric mice have been generated by bone marrow transplantation as previously reported[4]. Together with MMTV-PyMT/cathepsin $C^{-/-}$ (n=50) and heterozygous control mice (MMTV-PyMT/cathepsin $C^{+/-}$; n=50), we are assessing characteristics of neoplastic progression, e.g., tissue remodeling, angiogenesis, inflammation, epithelial hyperproliferation and differentiation, cancer incidence and metastasis. These analyses will define which parameters of malignancy are regulated by cathepsin C emanating from epithelial cells versus myeloid cells and if that regulation translates into altered overall primary cancer risk or development of metastases.

ACTIVATED MACROPHAGES DISRUPT MAMMARY EPITHELIAL CELL POLARITY BY A METALLOPROTEASE-DEPENDENT MECHANISM. We have hypothesized that infiltrating immune cells stimulate tumor progression in part by disrupting the polarity of mammary ductal epithelium which otherwise suppresses aberrant MEC proliferation. Utilizing organotypic 3-dimensional culture models[22] where MCF10A immortalized human mammary epithelial cells (hMEC) or MMTV-neu primary murine MECs (mMECs) are co-cultured with either naïve or activated leukocytes, we are assessing the effects of co-culture of specific inflammatory cells on mammary epithelial acini (ductal structure) polarity, proliferation, and apoptosis. Our preliminary findings indicate that conditioned medium from PMA-stimulated THP1 monocytes (macrophages), but not unstimulated THP1 monocytes, disrupts mammary acini polarity leading to invasion, an event that is inhibitable by addition of a broad spectrum metalloprotease inhibitor GM6001. Moreover, when primary mMECs are similarly cultured and 3D acini allowed to form, co culture with MMTV-PyMT tumor-derived macrophages but not spleen-derived macrophages similarly disrupts mMEC acini polarity and induces invasive growth characteristics, together indicating that macrophages, educated by their microenvironment, communicate with MECs and promote neoplastic growth properties via proteolytic mechanisms. We believe that studying the molecular mechanisms by which infiltrating immune cells stimulate the progression of premalignant lesions to undergo malignant transformation, we will identify intracellular and pericellular pathways that can be targeted for treatment and prevention of breast cancer.

DEVELOPMENT OF A NOVEL SPECT IMAGING AGENT: Targeted cancer radiotherapy requires highly selective radiolabeled carriers that provide efficient radionuclide delivery. Radiotherapeutics incorporating sophisticated selective cell-targeting strategies are promising, but few if any current radiotherapeutics have an optimal balance of long intra-tumoral residence times and adequate clearance from nontarget sites, and many suffer *in vivo* catabolism or metabolism of the radionuclide delivery molecule.

In order to selectively target a tumor, deliver a cytotoxic dose of radioactivity to tumor cells, and minimize non-specific radiation to non-tumor tissues, in collaboration with our colleague Dr. Benjamin Franc in the Department of Radiology (UCSF), we are developing a radiotherapeutic using a novel targeting strategy. The radionuclide delivery molecule (RDM) developed in this project is targeting matrix metalloproteinase-14 (MMP14), a protease that is up-regulated in many breast cancers. Each probe contains a poly-D-arginine cell-penetrating peptide, attached to an MMP14 cleavable peptide sequence, and a negatively-charged attenuation sequence. In our rational design approach, we employed an *in silico* screen to map the equilibrium conformations of four tested probe candidates, using an atomistic AMBER7 force field combined with GB/SA implicit solvation and modern conformational sampling techniques. In addition, we performed parallel synthesis of four probes and incorporated a bis-pyridyl chelate for labeling of ^{99m}Tc. Cleavage of these probes by MMP14 and the generation of fragments capable of translocation across a cell membrane have been demonstrated in cultured breast carcinoma cells. Molecular modeling was found to predict the efficiency of cell-penetrating peptide attenuation and protease cleavage of the peptide substrate. Currently, our work focuses on determining the pharmacokinetics of our probe, and whether tissue-selective accumulation of the radiolabeled probe can be detected *in vivo* by SPECT. The goal of this project is to demonstrate that the RDM, described above, is capable of delivering a therapeutic dose of radioactivity to a tumor. Following this initial proof of the proposed RDM strategy, the RDM will be tailored to target cancer cells, e.g., neoplastic cells and/or stromal cells within the tumor, that specifically express other proteases we are currently validating as functionally significant for tumor progression.

II. MIRCROENVIRONMENTAL REMODELING REGULATES TISSUE HOMEOSTASIS AND TUMOR PROGRESSION

Organs are endowed with innate regulatory networks that facilitate rapid responses to various forms of tissue assault. Such responses encompass programs regulating epithelialization, inflammation, vascular homeostasis **and** extracellular matrix (ECM) **remodeling - processes that are important for repair** and resolution of acute tissue damage and that are critically involved in the pathogenesis of several chronic disease states including cancer. We hypothesized that ECM molecules and the enzymes that remodel them are not only critical for regulating and resolving acute tissue damage, but also are molecules that regulate critical aspects of tissue homeostasis and cancer development. To address this hypothesis, we have taken a genetic approach and utilized mouse models of acute vascular activation, and skin and breast cancer development to identify important proteolytic enzymes and ECM molecules that represent functionally significant regulators of acute and chronic response pathways.

STROMAL CONTEXT REGULATES VASCULAR HOMEOSTASIS: Assembly and maintenance of mature tubular networks for blood circulation is crucial to all mammals. The three major components of these networks are endothelial cells, vascular smooth muscle cells (VSMCs) and ECM. Under homeostatic conditions, these networks are maintained in quiescent states. Following physiologic or pathologic stimulation however, innate programs are rapidly activated that foster vascular remodeling. Despite many advances in the fields of vasculogenesis and angiogenesis[28], relatively little is known about the molecular and cellular mechanisms that regulate vessel stability, leakiness or extravasation of plasma proteins into interstitial tissue compartments following acute stimulation[29,30]. Multiple factors likely influence the process including bioavailability of soluble factors that effect vascular cell physiology, luminal surface area, integrity of vessel walls, hydrostatic and osmotic gradients across endothelium, and the context of ECM in perivascular stroma.

We hypothesized that molecules embedded within ECM mediate these responses, in part by regulating physiological responses to external challenge/assault. Since perivascular stroma is rich in type I collagen-containing fibrils, we assessed the functional significance of type I collagen as a regulator of vascular homeostasis and acute response. Using mice harboring a double point mutation in the collagen α1(I) (*Col1aI*) gene, e.g., Collα1(I)$^{r/r}$ mice[31], that renders type I collagen fibrils resistant to cleavage by vertebrate type I collagenases *in vitro* and alters dynamics

of type I collagen metabolism *in vivo*, we identified a novel pathway regulated by type I collagen mediating acute vascular response[32]. Whereas plasma proteins rapidly extravasate out of vasculature in wildtype mice following cutaneous exposure to mustard oil, serotonin or VEGF, plasma protein extravasation is significantly restricted in Colα1(I)$^{r/r}$ mice following similar exposure. Appropriate vascular response and plasma protein leakage is restored in Collα1(I)$^{r/r}$ mice when mice are treated with neutralizing antibodies to TGFβ, ALK5 kinase inhibitors or broad-spectrum metalloproteinase inhibitors prior to challenge. Moreover, we found that steady-state plasma protein extravasation into interstitium is enhanced in wildtype mice following systemic treatment with ALK5 kinase inhibitors and as well as in mice harboring homozygous deletions in the *MMP14* gene. Together, these data indicate that perivascular type I collagen fibrils are important sensors that regulate vascular responses to tissue damage, not by extrinsically maintaining tissue architecture, but instead by regulating activity of cell surface metalloproteases and bioavailability of TGFβ that in turn regulate vascular homeostasis and acute vascular responses following tissue injury. Clinical ramifications of these results and identification of this novel pathway suggest that strategies targeting type I collagen metabolism, TGFβ bioavailability or TGFβ-regulated signaling may have therapeutic efficacy in regulating extravasation of molecules, e.g., plasma proteins and/or drugs, from the vasculature into interstitial tissue compartments. Current work is focused on, *i.* identifying molecular mechanisms whereby type I collagen regulates MMP14 activity on perivascular and/or endothelial cell membranes and how that in turn regulates TGFβ bioavailability and intracellular signaling, and *ii.* investigating the impact of pharmacologic manipulation of this pathway on drug delivery into fibrotic tissues and tumors.

TISSUE MICROENVIRONMENTS REGULATE NEOPLASTIC PROGRAMMING AND MALIGNANT OUTCOME DURING EPITHELIAL CARCINOGENESIS: Previous studies using HPV16 transgenic mice have demonstrated that some members of the matrix metalloproteinase (MMP) family represent functionally significant paracrine regulators of neoplastic progression[4,33] and suggest that molecules downstream of protease action play fundamental roles in carcinogenesis. We hypothesized that altering the susceptibility of functionally significant protease substrates to proteolytic attack may likewise alter and/or regulate neoplastic progression. Since type I collagen is an key metalloprotease substrate, we tested the hypothesis that during neoplastic progression, equilibrium between synthesis, accumulation, and degradation of type I collagen is rate-limiting for the cascade of cellular and genetic changes unfolding during skin carcinogenesis. Using HPV16 transgenic mice harboring a knock-in mutation in the endogenous *COL1A1* gene[31], e.g., HPV16/ColαI(I)$^{r/r}$, resulting in production of collagenase-resistant type I collagen fibers, we found that type I collagen remodeling is a functionally significant parameter of neoplastic programming that not only regulates the incidence of carcinomas that develop in HPV16, but also regulates the types of carcinomas that emerge from neoplastic epidermis. We utilized pathologic and genomic (array comparative genomic hybridization) studies to examine the characteristics of neoplasia in type I collagen mutant HPV16 mice and revealed that the context of the neoplastic microenvironment asserts selective pressure on evolving neoplasms and selects for carcinomas with distinctive genomic and phenotypic characteristics (manuscript in preparation).

REGULATION OF CUTANEOUS ANGIOGENESIS BY MMP-9: MMP-9 has emerged as an important paracrine regulator of tumor development largely due to its ability to regulate tumor angiogenesis by modulating bioavailability of vascular endothelial growth factor (VEGF) isoforms and thereby indirectly influencing neoplastic cell hyperproliferation, malignant conversion and cancer development. In mice predisposed to squamous carcinoma development , e.g., HPV16 transgenic mice, MMP-9-deficiency results in attenuated ability of keratinocyte to attain hyperproliferative growth characteristics, delayed development of angiogenic vasculature (as evidenced by the total percentage of CD31$^+$ endothelial cells present within neoplastic tissue), delayed recruitment of infiltrating leukocytes into dermal stroma, restricted accumulation of tissue fluid (TF) volume, and a 50% decreased incidence of squamous carcinomas[4]. To determine the degree to which altered features of cancer development in HPV16/MMP9$^{-/-}$ mice are due to decreased bioavailability of $VEGF_{164}$ and limited activation/expansion of angiogenic vasculature, we generated bigenic HPV16/MMP9$^{-/-}$ mice that express $VEGF_{164}$ in epithelial keratinocytes, e.g., HPV16/$VEGF_{164}$/MMP-9-deficient mice. Our data indicate that transgenic expression of $VEGF_{164}$ is sufficient to restore characteristic latency, development and functionality to angiogenic vasculature and to reinstate keratinocyte hyperproliferative growth characteristics in HPV16 mice. However, characteristics of leukocyte recruitment into premalignant skin of HPV16/MMP9$^{-/-}$ mice is not restored by bigenic expression of $VEGF_{164}$. Taken together these studies indicate that MMP9 regulates premalignant development of angiogenic vasculature and subsequent epithelial tissue expansion in part due to its ability to mobilize latent VEGF, but also reveal that infiltration and activation of leukocytes is VEGF-independent. We are currently investigating differential effects of MMP-9 and VEGF on mobilization of bone

marrow-derived cells and regulation of MMP-9-dependent leukocyte chemo-attractants that may participate in leukocyte recruitment during epithelial carcinogenesis.

UNIQUE RESPONSE PATHWAYS ARE ENGAGED BY BLOOD AND LYMPHATIC ENDOTHELIAL CELLS DURING EPITHELIAL CANCER DEVELOPMENT. Two interdependent vascular systems are present in mammals, e.g., blood and lymphatic vessels. Whereas blood vasculature delivers blood cells, plasma proteins and oxygen to tissues, lymphatic vessels constantly monitor interstitial fluid pressure and remove excess interstital fluid and debris to maintain tissue homeostasis[28,34]. During cancer development each vascular system undergoes distinctive architectural and physiologic changes that are likely preprogrammed to each organ microenvironment[28]. While it is well established that tumor development depends on the ability of pre-existing hematogenous vascular beds to undergo angiogenic activation[34], the role of tumor-associated lymphangiogenesis and changes unique to lymphatic vessels during cancer development is less clear. In an attempt to understand unique versus common functional and architectural features of blood versus lymphatic vascular networks that accompany and/or contribute to cancer development, we employed K14-HPV16 mice[3]. Skin on HPV16 mice develops epithelial hyperplasias by 1-month of age that progress into angiogenic dysplasias by 4 months out of which emerge squamous cell carcinomas (SCC) in 50% of mice. Hematogenous vasculature initiates angiogenic activation paralleling appearance of epithelial hyperplasias and is characterized by incremental increases in proliferation of $CD31^+$/$podoplanin^-$/$LYVE\text{-}1^-$ blood endothelial cells (ECs) at each stage of neoplastic development. In contrast, proliferation of $CD31^+$/$podoplanin^+$/$LYVE\text{-}1^+$ lymphatic ECs is enhanced as compared to nontransgenic skin, remains constant throughout premalignant progression, and significantly increases following malignant conversion and outgrowth of carcinomas. Increased vasodilation and vessels with 'open' lumens were characteristic of both blood and lymphatic networks in premalignant stroma; however, vessel sprouting is only found to be a common feature of angiogenic blood vessels and is not observed in lymphatic capillaries or collecting lymphatics. Blood vessel leakiness and tissue fluid (TF) volume is increased in premalignant stroma, whereas vessel leakiness decreased in carcinomas. Since lymphatic vessels in premalignant tissue efficiently clear tracer compounds, thus demonstrating their functional capacity, we hypothesized that high interstitial fluid pressure (IFP), a common feature of solid tumors, restricts fluid clearance capacity of collapsed carcinoma-associated lymphatics. Elevated IFP in tumors likely contributes to further elevation in TF volume in expanding tumors as tumor-associated blood vasculature becomes destabilized, further enhances IFP, resulting in a further restriction in blood vessel leakiness. We are currently evaluating accompanying changes in IFP that we predict will be elevated in carcinomas as opposed to premalignant stroma. Taken together, these data indicate that ECs composing both blood and lymphatic vascular systems similarly respond to altered microenvironmental conditions and pro-growth factors present during neoplastic development; however, unique physiologic responses are engaged by each vascular system manifesting distinct phenotypic and functional properties associated with cancer development.

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Inflaming Gastrointestinal Oncogenic Programming

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The etiology of gastrointestinal tumors implicates a role for chronic inflammation in response to pathogenic microflora as a promoting force for full neoplastic progression. Recently, Oguma and coworkers (2008) demonstrated that TNFα, derived from recruited macrophages, potentiates Wnt/β-catenin signaling and gastric carcinogenesis by activating Akt signaling and GSK3β phosphorylation independent of the NF-κB pathway in initiated epithelial cells. These observations provide a missing link in the mechanism whereby chronic inflammation, in response to *Helicobacter*, regulates the "penetrance" of initiating oncogenic mutations in the gastrointestinal tract leading to gastrointestinal tumorigenesis.

Epidemiologic studies have long supported a link between chronic inflammation and development of solid tumors (Coussens and Werb, 2002; Thun et al., 2004). More recently, through utilization of immunocompetent mouse models of multistage carcinogenesis, the molecular mechanisms whereby chronic engagement of the immune system (inflammation) potentiates development of epithelial cancers have begun to be elucidated (Balkwill et al., 2005; de Visser et al., 2006; Karin et al., 2006). Missing, however, has been insight into which soluble mediators derived from chronically activated immune cells are significant for regulating the penetrance of neoplastic cells harboring initiating mutations. For example, in the gastrointestinal tract, malignancy is frequently preceded by chronic inflammation sometimes associated with *Helicobacter pylori* infection (Blaser, 2000) or in individuals harboring activating mutations in the *APC* or *CTNNB1* genes or enhanced activation of Wnt/β-catenin signaling (Clements et al., 2002).

APC is a multifunctional cytoplasmic protein whose gene is frequently mutated in several types of gastrointestinal cancers. APC regulates both genomic instability and hyperactivation of the Wnt/β-catenin signaling pathway, and nuclear β-catenin accumulation is found in colon carcinoma cells at invasive fronts (Fodde and Brabletz, 2007). Recent experimental studies have revealed that malignant conversion of initiated *APC* mutant cells is potentiated by chronic activation of the Wnt/β-catenin signaling pathway (Fodde and Brabletz, 2007); however, links between these molecular mediators and chronic inflammation have not been previously identified.

Oguma and coworkers (2008) investigated this link and found that both enhanced Wnt expression and infection by gastric microflora induce submucosal infiltration by macrophages secreting high levels of tumor necrosis factor-alpha (TNFα). Binding of TNFα to TNF receptors on gastric epithelial cells enhances Akt phosphorylation that in turn induces glycogen synthase kinase 3β (GSK3β) phosphorylation, resulting in stabilization and nuclear accumulation of β-catenin that potentiates gastric carcinogenesis (Figure 1). To reveal this pathway, the authors utilized a mouse model of gastric tumorigenesis in which Wnt1 is expressed in gastrointestinal mucosal epithelia, K19-Wnt1 transgenic mice, which develop sporadic dysplastic lesions in the glandular stomach, mimicking hyperactivation of Wnt/β-catenin signaling commonly observed in patients with gastric carcinoma

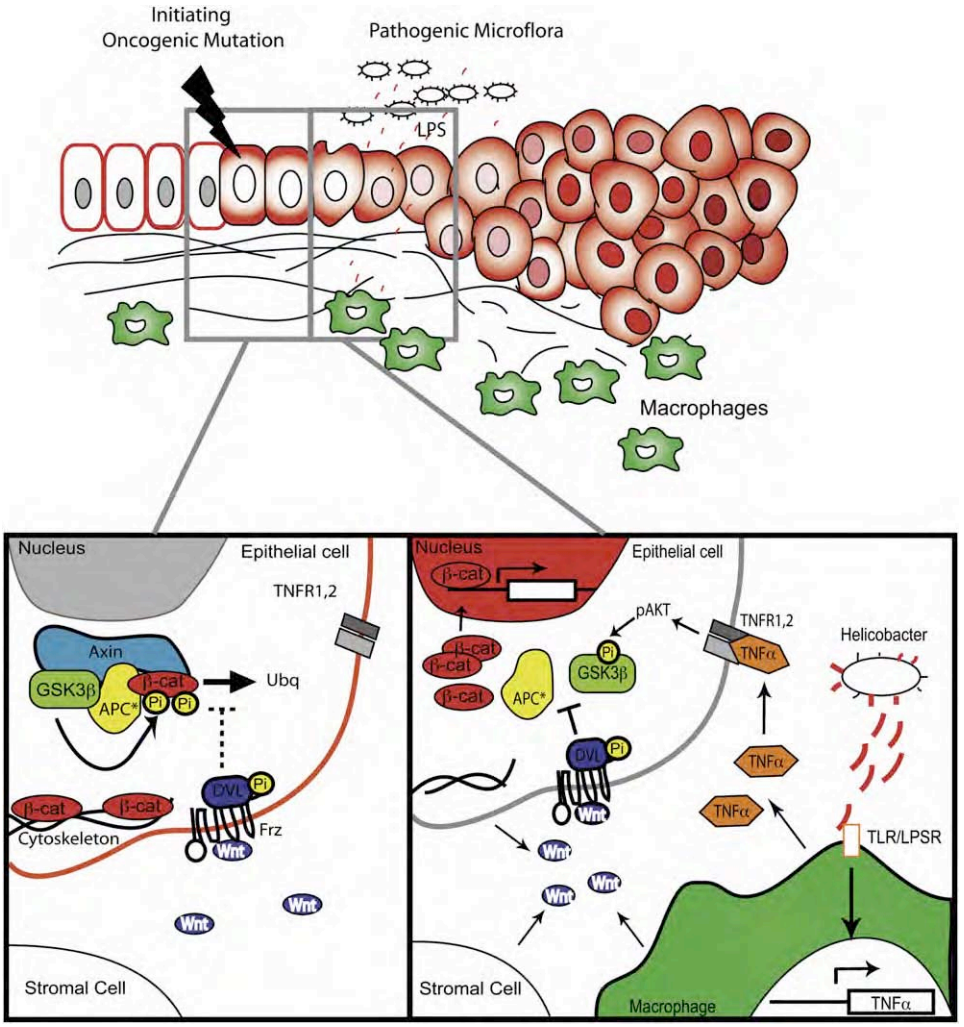

Figure 1. Macrophage-Derived TNFα Activates Wnt/β-Catenin during Gastrointestinal Cancer Development
Oncogenic mutations in tumor suppressors such as *APC* (APC*) are often associated with hyperactivation of Wnt/β-catenin pathways and stabilization of β-catenin. However, when such mutations occur in gastrointestinal cells, additional stimuli are required for translocation of β-catenin to the nucleus. Chronic inflammatory responses to pathogenic microflora such as *Helicobacter* can provide these stimuli via inducing macrophage infiltration. In response to bacterial components such as LPS or TLR ligands, macrophages produce TNFα, which in turn promotes nuclear accumulation of dephosphorylated β-catenin mediated by phosphoregulation of Akt and GSK3β in neoplastic epithelial cells in an NF-κB-independent manner. This process can be further exasperated by production of Wnt ligands by stromal cells that bind Frizzled receptors, leading to inhibition of the β-catenin degradation complex (APC, AXIN, GSK3β), thereby enabling nuclear translocation of stabilized β-catenin and transcriptional activation of Wnt target genes.

(Oshima et al., 2006). In K19-Wnt1 mice, macrophages accumulate in dysplastic areas of the glandular stomach adjacent to epithelia with nuclear accumulation of β-catenin, thus supporting a potential role for macrophages in regulating Wnt/β-catenin signaling. To functionally address this, the authors investigated a second model of gastrointestinal tumorigenesis, $Apc^{\Delta 716}$ mice, in which activation of Wnt/β-catenin signaling leads to development of intestinal polyps. By intercrossing $Apc^{\Delta 716}$ mice with *op/op* mice that have impaired colony-stimulating factor 1 (CSF1) signaling and decreased macrophage infiltration in tissues, the authors found a significant reduction in intestinal polyp formation, reduced levels of TNFα, and decreased Wnt/β-catenin activity that was largely due to inhibition of GSK3β and reduced Akt phosphorylation in gastric cancer cells.

To reveal whether this pathway is potentiated by infection, *Wnt1* transgenic mice were infected with *Helicobacter felis*—inflamed mucosa was histologically distinct from that found in dysplastic lesions of noninfected gut. Infected animals rapidly exhibited increased β-catenin accumulation in proliferating epithelial cells and decreased presence of H^+/K^+-ATPase-positive parietal cells, thus indicating that infection and inflammation promote Wnt/β-catenin signaling and suppress epithelial differentiation. Taken together, the results of the study by Oguma and coworkers (2008) reveal a new pathway whereby chronic infection of the gastrointestinal tract and the ensuing inflammatory microenvironment facilitate expansion of initiated cells by collaborating with existing oncogenic mutations in epithelia.

The tumor-promoting capability of macrophages has previously been demonstrated using a mouse model of

mammary carcinogenesis in which infiltration of macrophages into premalignant mammary tissue regulates mammary tumorigenesis (reviewed in Lin and Pollard, 2007). Failure to recruit macrophages into malignant tissue significantly delays development of late-stage carcinomas and attenuates pulmonary metastasis, likely via angiogenic mechanisms and altered paracrine interactions between epidermal growth factor (EGF) and CSF1 signaling pathways (Lin and Pollard, 2007). In breast and ovarian cancer, TNFα provided by tumor-infiltrating macrophages also promotes proinvasive phenotypes of neoplastic cells as well as potentiating metastatic spread in ovarian and hepatocellular cancer (Balkwill et al., 2005). The present study by Oguma et al. (2008) describes an additional tumor-promoting role for macrophages, independent of NF-κB-regulated pathways in epithelia, by providing a link between the proinflammatory cytokine TNFα and Wnt/β-catenin signaling.

In view of recent insights into mechanisms whereby microbial infections promote tumor development (Karin et al., 2006), it is intriguing that *Helicobacter*-associated inflammation promotes Wnt/β-catenin activity in initiated gastric epithelium since it raises the possibility that immune mediators directly influence stem cell niche expansion or "stemness" during gastric tumorigenesis. Cells of the gastrointestinal epithelium have a high turnover rate (3–6 days) whereby the balance between apoptosis, senescence, proliferation and differentiation is tightly regulated to maintain tissue homeostasis. Gastrointestinal epithelia are regenerated from stem cell populations residing in specific niches within the epithelium. Due to their relatively rapid turnover rate, differentiated gastrointestinal epithelia are not believed to persist long enough to acquire multiple genetic mutations that might be necessary for full malignancy before being shed into the lumen and/or undergoing apoptosis; thus, it has been postulated that gastrointestinal malignancies originate from stem cell populations. Wnt-regulated pathways are important for homeostatic stem cell renewal and are frequently upregulated during gastrointestinal carcinogenesis (Fodde and Brabletz, 2007). In addition to TNFα, macrophages have also been shown to directly produce Wnt ligands, including Wnt2 and Wnt5a in human colorectal tumors, further supporting a link between macrophages and activation of Wnt signaling pathways (Smith et al., 1999). It is unclear from the Oguma et al. (2008) study whether expression of Wnt ligands is also upregulated in stromal cells following *Helicobacter* infection, or whether Wnt ligands derived from macrophages act synergistically with TNFα to upregulate Wnt/β-catenin signaling. Considering the importance of Wnt signals during gastric carcinogenesis, it is tempting to speculate that chronically engaged immune cells may play a role in stem cell niche expansion during gastric tumorigenesis. In this manner, stem cell populations harboring initiating oncogenic or tumor suppressor gene mutations, such as *APC*, when present in a chronic inflammatory microenvironment might be subject to promotion-type events involving TNFα and/or Wnt ligands, leading to tissue expansion without differentiation, and in so doing might provide an expanded stem cell or stem cell-like population at risk for acquiring secondary tumor-promoting mutations. Thus, the findings of Oguma et al. (2008) provide a mechanism whereby *Helicobacter*-associated inflammation may facilitate expansion of initiated stem cells by promoting Wnt/β-catenin signaling and thereby promoting full neoplastic progression of initiated cells by nourishing the roots of cancer development.

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Chapter 20
Immune Cells and Inflammatory Mediators as Regulators of Angiogenesis

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Keywords: angiogenesis, cancer, inflammation, immune cells, leukocytes, monocytes.

Innate and Adaptive Immune Cells in the Initiation and Promotion of Tumorigenesis

During the past 25 years, cancer research has primarily focused on the role of activating and/or inactivating mutations in genes involved in cell proliferation, growth and death, and the ever-growing knowledge of genetic and epigenetic changes that occur in tumor cells, that together have led us to view cancer as a genetic disease. Moreover, the sequential activation/inactivation of a relatively low number of molecular pathways, common to many cancer types, has put forward the 'multi-step model of tumorigenesis', that regards the progressive accumulation of genetic changes in somatic cells as the major process in the etiology of cancer (Hanahan and Weinberg, 2000). However, although generally applicable, the multi-step model of tumorigenesis overlooks the role of the microenvironment in the development of cancer. Both experimental and clinical studies have recently highlighted the causal role of host-derived, extrinsic factors—such as the extra-cellular matrix (ECM), soluble molecules and tumor-associated cells—in the initiation and/or progression of cancer (Balkwill et al., 2005; Balkwill and Mantovani, 2001; Bissell and Radisky, 2001; Blankenstein, 2005; Coussens et al., 2000; Coussens and Werb, 2002; Joyce, 2005; Mueller and Fusenig, 2004). Solid tumors contain both neoplastic and non-neoplastic stromal cells, the latter of which not only passively support neoplastic cells by providing a scaffold for their growth, but also promote neoplastic development and regulate progression to malignancy. Cellular components of tumor stroma include (myo)fibroblasts, vascular cells and infiltrating leukocytes. The fact that some leukocytes promote—rather than restrict—tumor growth may be viewed as an apparent paradox (de Visser et al., 2006; Zou, 2005). Historically, leukocytes found in and around developing tumors were thought to represent an attempt by the host to eradicate transformed cells. Undeniably, certain leukocytes, such as some T lymphocyte subsets and natural killer (NK) cells, play a vital function in constraining tumor development (Dunn et al., 2004), and it has been postulated that many more tumors arise than those that eventually develop to fully malignant disease thanks to such activity. However, a growing body of research has recently implicated tumor-infiltrating leukocytes as causal players in cancer development (Balkwill et al., 2005; Balkwill and Mantovani, 2001; Coussens and Werb, 2002; de Visser et al., 2006; Karin, 2005, 2006).

Leukocytes, represent a diverse assortment of immune cells that can be divided into innate (myeloid) and adaptive (lymphoid) lineages. Innate immune cells, including macrophages, granulocytes, mast cells, dendritic cells (DCs) and NK cells, represent the first line of defence against pathogens and foreign agents. When tissue homeostasis is perturbed, tissue-resident macrophages and mast cells locally secrete soluble factors, such as bioactive mediators, matrix-remodelling proteins, cytokines and chemokines, that recruit additional leukocytes from the circulation into the damaged tissue, a process known as inflammation. The recruited innate immune cells (also referred to as inflammatory cells) can directly eliminate pathogenic agents in situ. DCs, on the other hand, take up foreign antigens (including tumor antigens) and migrate to lymphoid organs where they present their antigens to adaptive immune cells. Upon recognition of a foreign antigen presented by DCs or other professional antigen-presenting cells, adaptive immune cells, e.g., $CD4^+$ T lymphocytes and B lymphocytes, undergo clonal expansion and mount an "adaptive" response targeted to the foreign agent. Thus, acute activation of innate immunity sets the stage for the activation of a more sophisticated, antigenically

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committed adaptive immune response. Once the foreign agent has been eliminated, inflammation resolves and tissue homeostasis is restored.

The aforementioned processes continuously eliminate myriads of pathogens from our bodies while also implicated in antagonizing growth and expansion of sporadic tumors. However, the inflammatory response required for commencing immune responses may also set the ground for promoting neoplastic disease. As early as 1863, Virchow first postulated that cancer originates at sites of chronic inflammation, in part based on his hypothesis that some classes of irritants that cause inflammation also enhance cell proliferation. When tissues are injured or exposed to chemical irritants, damaged cells are removed by induction of cell death pathways, while cell proliferation is enhanced to facilitate tissue regeneration or wound healing, in the attempt to re-establish tissue homeostasis. Proliferation and inflammation resolve only after the insulting agent is removed or tissue repair completed. By contrast, when the insulting agent persists over time, sustained cycles of cell proliferation and death in environments rich in inflammatory cells and their bioactive products may increase neoplastic risk and/or foster tumor progression (Vakkila and Lotze, 2004). Thus, while sporadic or inherited genetic mutations in critical genes regulating cell cycle, differentiation, metabolism and cell adhesion may represent the initiating events in tumorigenesis ("initiation"), chronic inflammation then favors the selection of additional features in initiated cells and may promote their full malignant transition ("promotion").

It has been estimated that more than 15% of malignancies worldwide can be attributed to chronic inflammatory disease (Coussens and Werb, 2002; Finch and Crimmins, 2004). Perhaps the most compelling clinical evidence for a causative link between chronic inflammation and cancer comes from epidemiological studies reporting that inhibiting chronic inflammation in patients with pre-malignant disease, or who are predisposed to cancer development, has chemo-preventative potential (Dannenberg and Subbaramaiah, 2003). These studies revealed that long-term usage of anti-inflammatory drugs, such as aspirin and selective cyclooxygenase-2 (COX-2) inhibitors, significantly reduces cancer risk, indicating that COX-2 or other key molecules involved in prostaglandin biosynthesis might be effective anti-cancer targets.

Tumor microenvironments are rich in immune cell-derived cytokines, and growth factors, including tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), vascular endothelial growth factor (VEGF), and interleukins-1 (IL-1) and -6 (IL-6). These molecules not only foster tumour development via the modulation of gene expression programs in initiated neoplastic cells – culminating in altered cell cycle progression and enhanced survival – but also influence tumor-associated stromal cells and tissue remodelling. The availability of immune-competent mouse models of de novo carcinogenesis has facilitated a mechanistic evaluation of links between pre-malignant conditions, chronic inflammation and pro-inflammatory factors, with tumor progression. The transcription factor nuclear factor κB (NFκB), a mediator of cell survival, proliferation, and growth arrest, has been identified as an important molecule linking chronic inflammation to cancer (Balkwill and Coussens, 2004; Karin, 2006). Two studies (Greten et al., 2004; Pikarsky et al., 2004) revealed that the NFκB pathway promoted tumor development by a dual mechanism, by preventing apoptosis of cells with malignant potential, and by stimulating production of pro-inflammatory cytokines—including TNF-α—by innate immune cells. Pro-inflammatory cytokines then contributed to neoplastic cell proliferation in a paracrine fashion and increased survival and progression of initiated tumor cells. More recently, insight into the pathways linking innate and adaptive immunity in tumorigenesis have been provided, and have revealed that B lymphocytes and factors present in serum are essential for establishing chronic inflammatory states associated with pre-malignant progression in skin (de Visser et al., 2005; Mantovani, 2005). Antigens that are present in early neoplastic tissues, possibly derived from initiated neoplastic cells or stromal tissue components, may be transported to lymphoid organs by DCs, where antigen presentation triggers adaptive immune responses and B cell activation. Following B cell activation, immunoglobulins (Igs) are released in the circulation and accumulate at the site where antigens are expressed. Interstitial Ig deposition, that has been observed both in experimental and human pre-malignant tissues, can trigger the local activation/recruitment of innate immune cells (via the cross-linking of Ig receptors) and establish inflammation (Tan and Coussens, 2007). These observations linking Ig deposition to chronic inflammation not only establish a connection between initiation, adaptive and innate immune responses in cancer development, but also suggest that activating humoral immune responses in patients predisposed to cancer development might enhance neoplastic programming of tissue rather than preventing it.

Immune Cell-secreted Factors Promote Tumour Angiogenesis

In adulthood, most blood vessels are quiescent and angiogenesis—the growth of new blood vessels from pre-existing ones—only occurs during the female reproductive cycle and under certain pathophysiological conditions, such as tissue remodelling and wound healing. During angiogenesis, neo-vessels are formed through a well-orchestrated series of events, encompassing endothelial cell (EC) proliferation, as well as directional migration of ECs through remodelled basement membrane and toward angiogenic stimuli (Carmeliet, 2005). Once a primitive endothelial layer is formed, recruitment of perivascular support cells enables stabilization of nascent vessels, functional lumen formation and blood flow (Jain, 2003). In normal tissues, activation of proangiogenic molecular and cellular programs is regulated at many levels and controlled by a diverse assortment of positively and negatively acting soluble and insoluble mediators, whose balanced equilibrium is kept tightly in check under homeostatic conditions. However, under conditions of

tissue stress, such as those that occur during the onset of incipient neoplasia, this balance is rapidly upset, favoring proangiogenic programs (Bergers and Benjamin, 2003; Hanahan and Folkman, 1996). Whereas the cellular and molecular programs are common to both physiological and tumor angiogenesis, constitutively activated proangiogenic signaling in tumors make the tumor-associated vasculature distinctly irregular and chaotic in organization and inherently unstable, poorly functional and leaky (McDonald and Choyke, 2003; Morikawa et al., 2002).

There is a tight interplay between innate immune cells and the vascular system. ECs mediate immune cell recruitment to extravascular tissues by expressing a repertoire of leukocyte adhesion molecules. On the other hand, innate immune cells produce a number of soluble factors that influence EC behavior. In many physiological conditions, recruitment of an inflammatory infiltrate functionally supports angiogenesis and tissue remodelling (Fig. 20.1). During endometrial, decidual or retinal angiogenesis, inflammatory cells regulate vascular proliferation and patterning by producing both proangiogenic and antiangiogenic factors (Carmeliet, 2005; Gariano and Gardner, 2005; Girling and Rogers, 2005; Hanna et al., 2006; Ishida et al., 2003a, 2003b). Innate immune cells, e.g., granulocytes (neutrophils, basophils and eosinophils), DCs, macrophages, NK and mast cells, are also prominent components of pre-malignant and malignant tissues. They functionally contribute to cancer development by releasing of a myriad of cytokines, chemokines, matrix metalloproteinases (MMPs), serine proteases, DNA-damaging molecules (reactive oxygen species), histamine and other bioactive mediators that regulate cell survival, proliferation and motility, along with tissue remodeling and angiogenesis.

Some immune cell-secreted factors exert an important role in supporting tumor angiogenesis, a rate-limiting step for tumor development (Carmeliet, 2005; Carmeliet and Jain, 2000; Hanahan and Folkman, 1996). Tumor angiogenesis occurs in response to the increasing demand of nutrients and oxygen by the expanding neoplasm and, as early as 1971, Judah Folkman postulated that inhibition of angiogenesis would be an effective strategy to treat human cancer (Folkman, 1971). An active search for angiogenesis inducers and inhibitors began thereafter. Extensive research has identified several regulators of angiogenesis, some of which may represent therapeutic targets. Moreover, identification of immune cell-secreted angiogenic factors has put forward the concept that targeting tumor-infiltrating immune cells may represent a valuable anti-cancer strategy.

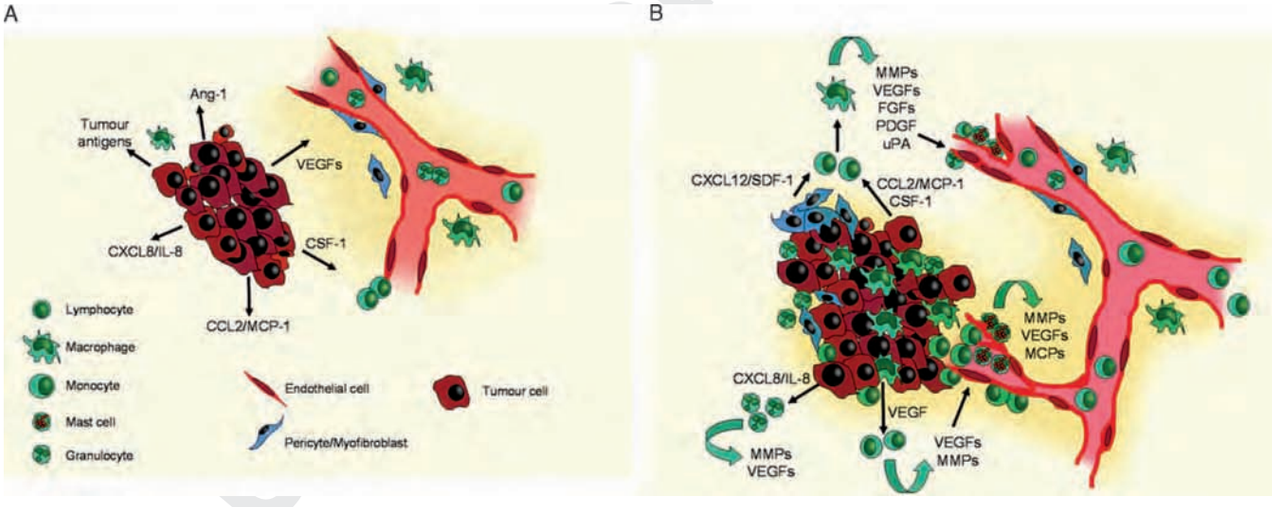

FIG. 20.1. Tumor-derived factors promote angiogenesis by recruiting proangiogenic hematopoietic cells. **A** Initiated neoplastic cells secrete factors that recruit different leukocytic populations, mostly myeloid-lineage cells, including *mast cells*, *neutrophils* and *monocytes*. These tumor-secreted factors include monocyte (colony-stimulating factor-1, *CSF-1*; monocyte chemoattractant protein-1, *MCP-1*; vascular endothelial growth factors, *VEGFs*) and neutrophil (inerleukin-8, *IL-8*) chemoattractants. Other tumor-secreted factors that likely recruit leukocytes are the Angiopoietins (*Ang-1*). Some of these factors also recruit vascular endothelial cells and thus directly stimulate angiogenesis (such as *VEGFs*). **B** The influx of leukocytes into premalignant tissues provides additional factors that foster tumor progression and angiogenesis. Some monocyte chemoattractants stimulate the differentiation of monocytes into macrophages. These cells, together with tumor-infiltrating neutrophils and mast cells, produce a wide array of proangiogenic factors, such as *VEGFs*, fibroblast growth factors (*FGFs*), matrix metalloproteinases (*MMPs*), platelet-derived growth factor (*PDGF*), mast cell-specific serine proteases (*MCPs*) and urokinase-type plasminogen activator (*uPA*). In addition, tumor-infiltrating mesenchymal cells, such as myofibroblasts, produce high levels of stromal cell-derived factor-1 (*SDF-1*), which further recruits $CXCR4^+$ leukocytes to tumors and stimulates the migration of endothelial cells engaged in the angiogenic process.

The Extra-cellular Matrix and Proteases in Tumor Angiogenesis

ECM proteins are produced by both epithelial and stromal cells, with epithelial cells typically contributing basement membrane components (including type IV collagen and laminin) and stromal cells, mainly fibroblasts, supplying connective tissue components, such as type I collagens (Kalluri, 2003). ECM strongly influences tumor development. Perturbation of ECM, either through proteolysis and degradation or altered deposition, can positively contribute to tumor progression in a significant way (Bissell and Radisky, 2001; Littlepage et al., 2005; Mueller and Fusenig, 2004; Tlsty, 2001; van Kempen et al., 2006). On the other hand, restoration of 'normal' cell-matrix interactions would supply "normalizing" signals to transformed epithelial cells. The matrix acts as a depot for a plethora of growth and proangiogenic factors, including basic fibroblast growth factor (bFGF), VEGF, transforming growth factor-β (TGF-β), and proteolysis of the matrix can unleash a powerful burst of frequently pro-tumorigenic signaling events. Proteolytically generated fragments of matrix molecules are themselves potent soluble signaling molecules, as is particularly evident with the antiangiogenic fragments angiostatin, endostatin, tumstatin, and others. Thus, the ECM may provide both pro- and antiangiogenic signals to incipient tumors.

ECM-degrading enzymes can be produced within tumor microenvironments by many cell types. Typically, these are proteases of the metallo-, serine and cysteine classes, but other enzymes, for example, heparanase, which is a glucuronidase, are also important (van Kempen et al., 2006). MMPs are a family of highly homologous, secreted or plasma membrane-associated zinc-binding proteinases (Bergers and Coussens, 2000; Egeblad and Werb, 2002). MMPs are produced by multiple cell types, regulate many developmental processes and participate in a variety of pathological conditions, including cancer. Initially, MMPs were thought to facilitate neoplastic progression by merely degrading ECM structural components, thereby allowing migration of tumor or ECs. Indeed, cleavage of collagen type I is required for EC invasion of ECM and vessel formation. Several proangiogenic growth factors, most notably VEGF, bFGF and TNF-α, are highly expressed in developing tumors. However, their bioavailability is limited, as they are either sequestered to ECM molecules or tethered to the cell membrane. MMPs (and other extracellular proteases) regulate release of these factors, rendering them available for interaction with cognate receptors on vascular cells and thus activating development of tumor-associated vasculature (Heissig et al., 2003). This function of MMPs was highlighted in mouse models of multi-step carcinogenesis. In the RIP1-Tag2 mouse model of pancreatic islet carcinogenesis (Hanahan, 1985), the angiogenic switch that promotes islet angiogenesis and progression to malignancy specifically occurs upon activation of VEGF receptor-2 (VEGFR-2) on ECs. Interestingly, Bergers and colleagues observed that VEGF and acidic FGF were both constitutively expressed in normal islet β-cells of control mice and in all stages of the RIP1-Tag2 islet carcinogenesis. However, these authors demonstrated that, during the angiogenic switch, cells expressing MMP-9/gelatinase-B infiltrated dysplastic islets and MMP-9 produced by tumor-infiltrating immune cells released matrix-sequestered VEGF, making it available for interaction with its receptor on ECs (Bergers et al., 2000). Coussens and colleagues similarly demonstrated that MMPs and serine proteases that promoted tumor angiogenesis and progression in the K14-HPV16 model of skin carcinogenesis (Arbeit et al., 1994) were produced by bone marrow (BM)-derived myeloid cells (Coussens et al., 1999, 2000). In one study (Coussens et al., 1999), infiltration by mast cells and activation of MMP-9 coincided with the angiogenic switch in pre-malignant skin lesions. Mast cells infiltrating hyperplasias, dysplasias, and invasive fronts of carcinomas were shown to degranulate in close apposition to capillaries and epithelial basement membranes, releasing MMP-9 and the mast cell-specific serine proteases MCP-6 (tryptase) and MCP-4 (chymase). MCP-6 is a mitogen for dermal fibroblasts that proliferate in the reactive stroma, whereas MCP-4 activates pro-MMP-9 and induces hyperplastic skin to become angiogenic. In this model, MMP-9 increased the rate and broadened the distribution of hyperproliferation of oncogene-expressing keratinocytes, enhancing malignant conversion of dysplasias into frank carcinomas, and affecting differentiation characteristics of emergent tumours. Notably, mast cell deficiency in K14-HPV16/KIT^{mut} mice resulted in a severe attenuation of early neoplasia, strengthening the notion that mast cells are functionally important in the angiogenic switch. In another study (Coussens et al., 2000), the transplantation of wild-type BM cells into K14-HPV16/MMP-9^{null} mice, ~~that had~~ decreased ~~the~~ incidence of skin tumors as compared to K14-HPV16 mice, restored angiogenesis and full neoplastic progression in the mutant mice. In this study, MMP-9 was shown to be predominantly expressed by BM-derived mast cells, monocytes/macrophages and neutrophils infiltrating both pre-malignant and malignant stages of skin carcinogenesis. Together, these studies have uncovered important regulatory capabilities for MMP-9, both during pancreatic and skin carcinogenesis. Importantly, MMP-9 imparted these regulatory capabilities on oncogene-positive neoplastic cells as a paracrine factor, originating from inflammatory cells conscripted to support neoplastic growth and progression.

The processing of pro-growth factors is not a unique property of MMP-9. In fact, several MMP family members are known to possess this property, and some of them (such as MMP-2, MMP-7, MMP-12) also regulate inflammation, tissue remodeling and angiogenesis through their ability to process ECM and ECM-embedded chemokines (Egeblad and Werb, 2002). A major role for cysteine cathepsin proteases as important mediators of angiogenesis and cancer development has also been recently appreciated (Gocheva et al., 2006; Joyce, 2005; Joyce et al., 2004). Cysteine cathepsins

are lysosomal proteases produced by epithelial cells and leukocytes. These enzymes are involved in many physiological and pathological processes, including tissue remodeling, epithelial homeostasis, degradation of ECM, cell migration and invasion, regulation of inflammatory and immune responses and activation of angiogenesis. Joyce and colleagues recently demonstrated association of increased cathepsin activity with angiogenic vasculature and invasive fronts of carcinomas during tumorigenesis in transgenic mouse models of pancreatic and cervical carcinogenesis (Gocheva et al., 2006; Joyce, 2005; Joyce et al., 2004). In particular, cysteine cathepsins C and H (among 11 members analyzed) were found to be specifically expressed by immune cells. Of note, the pharmacological inhibition of cysteine cathepsin activity impaired angiogenic switching, tumor growth, and invasion in ~~the~~ pancreatic tumor model, suggesting that broad-spectrum cysteine cathepsin inhibitors may effectively block multiple biological aspects of tumor development, offering new therapeutic opportunities in anti-cancer therapy.

As mentioned above, many matrix-remodeling proteases are stored within and rapidly released from tumor-infiltrating myeloid cells. It has been recently shown that amino-bisphosphonate-mediated blockade of MMP-9 production by macrophages significantly reduced cervical cancer development in K14-HPV16 mice (Giraudo et al., 2004). Thus, drugs that inhibit selected proangiogenic MMPs or other ECM-modifying enzymes should hold promise for effective anti-cancer therapies (Coussens et al., 2002). However, whereas remodeling of ECM or certain matrix-bound molecules confers a proangiogeneic phenotype (e.g., VEGF), remodeling of others may confer antiangiogenic properties (Sottile, 2004).

Embedded within some ECM molecules are bioactive cryptic protein fragments released by proteolytic cleavage. The first example of the release of a bioactive ECM fragment was the isolation of angiostatin from the urine of mice with Lewis lung cell carcinoma (O'Reilly et al., 1994). Angiostatin is a plasminogen cleavage product that inhibits EC proliferation and is thought responsible for maintaining Lewis lung cell metastases in a dormant state. Several MMPs, including MMP-2, -7, -9, and -12, can generate angiostatin. Another ECM fragment with antiangiogenic properties is endostatin, produced by cleavage of collagen type XVIII by MMP-3, -7, -9, -12, -13, and -20 and acts by inhibiting EC proliferation (O'Reilly et al., 1997).

In conclusion, MMP-generated cleavage products of ECM and soluble molecules act either as activators or suppressors of angiogenesis, often in tissue-dependent and stage-dependent manners, and implicate MMP-producing cells—immune cells in particular—as important mediators of tumor-associated angiogenesis ~~by both pro-tumour and anti-tumour mechanisms.~~ Thus, the bi-functional activity of MMPs and of other ECM remodelling enzymes in the context of tumor angiogenesis should be taken into account when designing anti-cancer therapies that selectively target their functions (Coussens et al., 2002).

Growth Factors, Chemokines and Cytokines in Tumor Angiogenesis

~~Several tumor-secreted~~ growth factors and chemokines are potent proangiogenic mediators (Fig. 1). ~~This function is often exerted through the activity or the recruitment of tumour-infiltrating leukocytes at the tumour site, although direct stimulation of ECs and vessel-associated cells is also possible.~~

VEGF family members and the MMP connection

VEGF family molecules are protypical proangiogenic factors (Alitalo and Carmeliet, 2002; Ferrara et al., 2003; Hicklin and Ellis, 2005; Hiratsuka et al., 2001; Luttun et al., 2002a; Shibuya, 2006; Shibuya and Claesson-Welsh, 2006). VEGF-A gene expression is up-regulated by hypoxia, a common feature of the tumour microenvironment. VEGF-A binds to two EC receptor tyrosine kinases, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1 or KDR). VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-inducing effects of VEGF-A on ECs. The role of VEGFR-1 in the regulation of EC biology and angiogenesis is more complex. The VEGFR-1, ~~that~~ is not uniquely expressed by ECs, but also by hematopoietic progenitors, monocytes/macrophages and BM stromal cells, can also function as a 'decoy' receptor that sequesters VEGF-A and prevents its interaction with VEGFR-2 (Eubank et al., 2004; Kendall et al., 1996). In addition to VEGF-A, the VEGF homologue placental growth factor (PlGF) also binds to VEGFR-1 and stimulates angiogenesis (Luttun et al., 2002a).

There is growing evidence that VEGFR-1 has important roles in hematopoiesis and in recruitment of monocytes and other BM-derived cells to tumors and ischemic tissues (Kopp et al., 2006; Luttun et al., 2002a; Shibuya, 2006). Tissue injury and tumor growth can indeed induce plasma elevation of both VEGF-A and PlGF, which promote chemotaxis of hematopoietic progenitors, their mobilization from the BM and recruitment to neo-angiogenic niches (Hattori et al., 2002; Luttun et al., 2002b). Interestingly, macrophages from VEGFR-1 deficient mice display significantly reduced migration in response to VEGF in a mouse model of embryonic angiogenesis (Hiratsuka et al., 1998). By using a genetic approach to switch expression of VEGF on and off in the heart or liver, Keshet and colleagues showed that an increase in VEGF expression in these organs induced robust angiogenesis (Grunewald et al., 2006). The authors found that locally expressed VEGF efficiently mobilized VEGFR-1$^+$ myeloid cells from BM and recruited them to the target organs, where they stimulated angiogenesis by producing proangiogenic factors. Although this study did not investigate tumor angiogenesis, it contributes to the emerging view that proangiogenic factors (such as VEGF) not only directly stimulate the local proliferation of ECs, but also recruit proangiogenic BM-derived hematopoietic cells at sites of angiogenesis (Ruiz de Almodovar et al., 2006).

Neuropilin-1 (NP-1) is a VEGF-A co-receptor expressed by ECs and hematopoietic cells that enhances VEGF signaling through VEGFR-2 (Lee et al., 2002). It has been recently proposed that the NP-1–VEGF complex on hematopoietic cells exogenously stimulates VEGFR-2 activation and induces brisk proliferation of ECs. According to this model, leukocytes expressing NP-1, such as monocytes/macrophages that often acquire a peri-endothelial position in tumors, may cluster the proangiogenic factor VEGF in the immediate vicinity of ECs, thus enhancing angiogenesis (Takakura, 2006; Yamada et al., 2003).

Besides a direct chemoattractant function, VEGF and PlGF can up-regulate MMP-9 expression in tissue stromal cells, including mesenchymal cells, ECs and leukocytes. Locally expressed MMP-9 then facilitates remodeling of the ECM, enhances cell migration (e.g., in tumours and BM microenvironment), and increases bio-availability of VEGF for ECs (Heissig et al., 2003; Hiratsuka et al., 2002). Rafii and colleagues have shown that BM suppression or plasma elevation of VEGF-A and PlGF results in a timely up-regulation of MMP-9 in the BM, that facilitated proteolytical release of bioactive soluble Kit ligand (sKitL). sKitL (also known as stem cell factor, SCF) then promotes proliferation and mobilization of BM c-Kit$^+$ hematopoietic progenitors (Heissig et al., 2002). These studies indicate that BM activation of MMPs may serve as a checkpoint for mobilization of myeloid progenitors from the BM to the peripheral circulation. Mobilized myeloid-lineage cells, that express VEGFR-1, are then recruited to VEGF-expressing tissues and tumors, where they promote angiogenesis in a paracrine fashion.

In summary, VEGF family members not only directly activate VEGFRs on ECs (thus enabling EC proliferation and angiogenesis), but also indirectly stimulate angiogenesis by (1) recruiting proangiogenic (VEGFR-1$^+$) inflammatory cells to tumors and other angiogenic tissues, and by (2) activating MMPs and facilitating tissue remodelling.

Stromal Cell-derived Factor-1

BM-derived and mesenchymal stromal cells, constituting a large proportion of the non-neoplastic cells found in tumors, secrete the chemokine stromal cell-derived factor-1 (SDF-1/CXCL12). SDF-1 secreted by stromal cells functions as a chemoattractant for cells expressing its cognate receptor, CXCR4, that is expressed by a broad range of cell types, including cancer cells, ECs, hematopoietic cells and their progenitors (Burger and Kipps, 2006). It has been recently highlighted that the SDF-1/CXCR4 axis promotes tumor progression by both direct and indirect mechanisms. In addition to stimulating the survival and migration of CXCR4$^+$ cancer cells, SDF-1 promotes tumor angiogenesis by attracting CXCR4$^+$ ECs and myeloid cells to the tumor microenvironment (Guleng et al., 2005; Kryczek et al., 2005). Weinberg and colleagues reported that carcinoma-associated (myo)fibroblasts isolated from breast cancers, but not normal tissue fibroblasts, secrete high levels of SDF-1, that enhanced tumor angiogenesis by functioning as a potent chemoattractant for locally derived ECs and BM-derived hematopoietic cells (Orimo et al., 2005). In addition, it has been shown that proangiogenic VEGFR-1$^+$ myeloid cells also express CXCR4 and that the up-regulation of SDF-1 by perivascular cells is a major determinant for recruitment and retention of proangiogenic VEGFR-1$^+$CXCR4$^+$ myeloid cells around nascent vessels (Grunewald et al., 2006; Jin et al., 2006). Accordingly, inhibition of the SDF-1/CXCR4 axis decreased the growth of experimental tumors through the suppression of tumor angiogenesis (Guleng et al., 2005). As such, CXCR4 antagonists, although initially developed for the treatment of non-neoplastic diseases, may actually become effective agents for the treatment of cancer.

Colony Stimulating Factor-1 and Monocyte Chemoattractant Protein-1

The recruitment of monocytes/macrophages to pre-malignant stages has been shown to promote angiogenesis and malignant progression (Lin et al., 2001; Lin and Pollard, 2004). Colony-stimulating factor-1 (CSF-1) is a key macrophage growth factor, responsible for the survival, proliferation, differentiation and chemotaxis of monocytes/macrophages. CSF-1 is broadly expressed by tumors of the reproductive system, and its expression was found to correlate with the extent of leukocyte infiltration and a poor prognosis in these tumours (Lin and Pollard, 2004). Pollard and colleagues have reported that in a Polyoma Middle T antigen-induced mouse model of mammary carcinogenesis (MMTV-PyMT), an increase of macrophage infiltration at the primary tumor site occurred immediately before the angiogenic switch and the onset of malignancy (Lin et al., 2006; Lin et al., 2001). By using PyMT mice carrying a *Csf-1* null mutation (*Csf-1*$^{op/op}$), these authors further demonstrated that depletion of CSF-1 markedly decreased infiltration of macrophages at tumor sites, and this inhibited the angiogenic switch and significantly delayed tumor progression. The knock-down of CSF-1 in transplanted tumour cells (by using antisense oligonucleotides) also resulted in inhibition of tumor growth, with tumors exhibiting extensive necrosis and poor vascularization, phenotypes that could be reversed by treatment of the mice with CSF-1. The premature macrophage infiltration in the mammary gland of MMTV LTR-CSF-1 transgenic mice induced robust angiogenesis even at early pre-malignant stages, providing evidence for a direct link between macrophage infiltration and angiogenesis, independent of tumor stage (Lin et al., 2006). These studies have provided evidence that CSF-1 is a major regulator of macrophage recruitment to tumors, and have shed light on the important roles of macrophages in tumor progression and in particular with tumor-associated angiogenesis.

Similar to CSF-1, several CC chemokines, particularly CCL2 (formally monocyte chemoattractant protein-1, or MCP-1) and CCL5 (RANTES, or regulated on activation normal T cells expressed and secreted), have been implicated in the recruitment of monocytes to tumors (Lin and Pollard, 2004; Murdoch

et al., 2004). CCL2/MCP-1 over-expression by genetically modified tumor cells implanted in mice promoted monocyte uptake by the tumor mass. In human tumors, CCL2/MCP-1 and CCL5/RANTES are mainly produced by tumor cells and fibroblasts, and their expressions have been shown to correlate with macrophage infiltration in many tumors, including bladder, cervix, ovary, breast, lung and brain cancers. Furthermore, both CCL2/MCP-1 and CCL5/RANTES were shown to stimulate monocyte/macrophage-lineage cells to secrete MMP-9 and urokinase-type plasminogen activator (uPA), which through their ECM-remodeling functions are potent activators of angiogenesis (Murdoch et al., 2004). Thus, thanks to their ability to attract proangiogenic monocytes/macrophages to tumors, both CSF-1 and CCL2/MCP-1 can be regarded as major players in the orchestration of the angiogenic process in tumors.

Interleukin-8

Apart from cell-autonomous effects in tumor cells, Ras oncogene expression regulates tumor-host interactions that are essential for neoplastic progression. Constitutive Ras activity promotes tumor cell invasiveness and angiogenesis by enhancing the expression of MMPs and VEGF, and by down-regulating expression of the antiangiogenic factor thrombospondin-1 (TSP-1). Bar-Sagi and colleagues have recently implicated Ras in inflammation-dependent angiogenesis (Sparmann and Bar-Sagi, 2004). These authors demonstrated that activation of Ras proto-oncogenes in cancer cells resulted in up-regulation of the inflammatory chemokine interleukin-8 (IL-8, also known as CXCL8), which in turn promoted tumor growth by enhancing leukocyte infiltration and angiogenesis. In tumors, IL-8 is a potent chemo-attractant for neutrophils that express the IL-8 receptors CXCR-1 and CXCR-2. The authors found that neutralization of IL-8 with antibodies inhibited tumorigenic growth of cancer cells xenografted in immunocompromised mice. Indeed, anti-IL-8 antibodies reduced recruitment of host innate immune cells, decreased tumor vascularization and slowed tumor growth. Although the precise identity of cell type targeted by the tumor-derived IL-8 was not identified in this study, and it is formally possible that IL-8 may directly activate ECs in the angiogenic process, one likely scenario is that angiogenesis was promoted by a product of inflammatory cells recruited to the tumor via IL-8-induced chemotaxis. Thus, IL-8 establishes a direct link between oncogene activation and inflammation in cancer (Karin, 2005). Moreover, IL-8 adds to the list of tumor-derived factors (VEGF, SDF-1, CSF-1, CCL2/MCP-1, CCL5/RANTES, and others) that indirectly promote angiogenesis by recruiting proangiogenic innate immune cells—mostly neutrophils and macrophages—to tumors.

Immune Cells Promote Tumour Angiogenesis

Myeloid-lineage immune cells, such as mast cells, macrophages and neutrophils, have been demonstrated to promote tumor progression by exerting a number of pro-tumoral activities, e.g., by stimulating angiogenesis (Coussens et al., 1999; De Palma et al., 2005; Nozawa et al., 2006; Okamoto et al., 2005; Takakura, 2006), suppressing anti-tumour immunity (Blankenstein, 2005; Bronte et al., 2006; Zou, 2005), and enhancing tumor cell migration and metastasis (Condeelis and Pollard, 2006; Wyckoff et al., 2004). Nucleated hematopoietic cells that have been directly implicated in tumor angiogenesis include mast cells (Coussens et al., 1999), tumor-associated macrophages (Balkwill et al., 2005; Lewis and Pollard, 2006; Pollard, 2004), Tie2-expressing monocytes (De Palma and Naldini, 2006; De Palma et al., 2005), neutrophils (Nozawa et al., 2006), dendritic cell precursors (Coukos et al., 2005) and myeloid immune suppressor cells (Serafini et al., 2006; Yang et al., 2004). Other hematopoietic cell types, such as platelets (Kisucka et al., 2006), eosinophils (Puxeddu et al., 2005) and hematopoietic progenitors (Takakura et al., 2000), also participate in angiogenic processes, but it remains to be established whether they can directly promote tumor angiogenesis, rather than having a broader function in supporting tissue inflammation and remodeling.

Mast Cells

Mast cells accumulate during the premalignant stages of tumor progression and at the periphery of invasive tumors, consistent with a role in activation of angiogenesis. Mast cells have direct proangiogenic activity owing to their production of MMPs, particularly MMP-9, and secretion of angiogenic factors, including basic FGF, VEGF, and IL-8. In addition, mast cells indirectly stimulate angiogenesis by secreting mast cell-specific serine proteases (MCP-4 and MCP-6) that activate pro-MMPs and stimulate stromal fibroblasts to synthesize collagens (Coussens et al., 1999; Ribatti et al., 2001). Mast cell-secreted VEGF can also up-regulate MMP-9 expression in ECs and other stromal cells and enhance the angiogenic response in the tumor microenvironment. In one study, the genetic deficiency of mast cells was shown to prevent the angiogenic switch and abate premalignant progression in a K14-HPV16/KIT-mutant mouse (Coussens et al., 1999). In addition to promoting angiogenesis, mast cells are a rich source of cytokines and chemokines, such as IL-1, IL-3, IL-4, granulocyte-macrophage colony-stimulating factor (GM-CSF), CCL-2/MCP-1, macrophage inflammatory protein (MIP-1)-α and β, TNF-α and interferon-γ. Many of these molecules contribute to the tumor microenvironment by enhancing tumor cell growth and invasion either directly or through cell intermediaries such as macrophages.

Tumor-Associated Macrophages (TAMs)

Many human tumors are infiltrated by macrophages, that may derive both from locally recruited tissue macrophages or, more likely, from circulating monocytes (Pollard, 2004). As discussed above, CCL2/MCP-1 and CSF-1 are well known chemoattractants for monocytes/macrophages in tumors. Despite the importance of macrophage infiltrates found in

human tumors, the biological significance and prognostic value of these infiltrates has been obscure for many years. Historically, activated macrophages were merely thought of as effector cells that phagocyte microorganisms and kill tumor cells. However, during the past 15 years many clinical reports have highlighted a correlation between the macrophage infiltration in tumors and a poor clinical prognosis (Lin and Pollard, 2004; Pollard, 2004).

It is known that TAMs heavily infiltrate necrotic areas in tumours, where they scavenge cellular debris. In addition, TAMs accumulate at hypoxic tumor areas, where they may cooperate with tumor cells to promote angiogenesis (Lewis and Murdoch, 2005; Murdoch et al., 2004). In fact, hypoxia stimulates expression of several proangiogenic molecules by activating hypoxia-inducible factors (HIFs) in TAMs. Expression of the monocyte chemoattracts VEGF, endothelin 2, and endothelial monocyte-activating polypeptide II (EMAP II) by hypoxic tumour cells can attract TAMs into hypoxic areas within tumors. It is believed that TAMs are then retained in hypoxic tumor areas due to abrogation of chemotactic signal transduction and the down-regulation of chemoattractant receptors. Once in hypoxic areas, TAMs produce a wide array of proangiogenic molecules and matrix-remodeling factors, including IL-8/CXCL8, VEGF, FGF, platelet-derived growth factor (PDGF), MMPs and uPA, but it remains to be clarified how crucial these TAM-secreted factors are in the economy of tumor angiogenesis, since many proangiogenic molecules are also produced by other components of the tumor stroma and by the tumor cells themselves. In addition, TAMs are characterized by a high degree of heterogeneity, and it may be difficult to establish whether TAMs in general, rather than specific subsets of these cells, play a critical role in tumor angiogenesis (Balkwill et al., 2005; Lewis and Pollard, 2006).

In addition to the aforementioned proangiogenic factors, TAMs release other molecules that can influence angiogenesis (Pollard, 2004). TAMs are key producers of TNF-α, that can up-regulate expression of thymidine phosphorylase (TP) and MMP-9. TAMs also produce IL-1, which may increase VEGF transcription by up-regulating expression of HIF-1- α through COX 2. In addition, TAMs also release nitric oxyde (NO), a molecule that provokes vasodilation and increased vascular flow, through the activity of inducible NO synthase (iNOS).

It has been proposed that many tumor-secreted factors, such as IL-4, IL-13, IL-10, CSF-1, TGF-β and prostaglandin E2, can blunt the tumoricidal activity of macrophages and activate them to acquire a growth-promoting and proangiogenic function. These "alternatively activated macrophages" are thought to represent a functional state within a continuum of macrophage phenotypes (Balkwill et al., 2005). Alternatively activated macrophages enhance the invasiveness of cancer cells by releasing growth factors and products of the arginase pathway; in addition, they promote EC proliferation and tissue remodelling. It has been proposed that classically activated macrophages—endowed with microbicidal and anti-tumor activity—and alternatively activated macrophages—endowed with growth-promoting and proangiogenic activity—differ in receptor expression, cytokine production and intracellular signaling (Balkwill et al., 2005). However, further studies are required to rigorously characterise these macrophage subsets *in vivo*.

Tie2-Expressing Monocytes (TEMs)

TEMs are a subset of circulating and tumor-infiltrating monocytes characterized by expression of the angiopoietin receptor Tie2 (De Palma et al., 2003), a molecule previously known to be restricted to ECs and haematopoietic stem cells (Jones et al., 2001). TEMs have been observed in several mouse tumor models—including subcutaneous tumor grafts, orthotopically growing gliomas and spontaneous pancreatic tumors—where they represent 1–15% of the total $CD11b^+$ myeloid cells. A peculiar feature of TEMs is that they preferentially localize around angiogenic blood vessels in tumors, a figure that is consistent with their marked proangiogenic activity in transplantation assays (De Palma et al., 2005). It appears that TEMs are a subpopulation of TAMs, possibly overlapping with alternatively activated macrophages (Balkwill et al., 2005). However, TEMs can be distinguished from other TAM populations by their surface marker profile, their preferential localization around angiogenic tumor vessels, their absence from necrotic tumour regions, and their marked proangiogenic activity.

TEMs can substantially accelerate vascularization of tumor grafts and are required for angiogenesis in certain tumors, thus they may represent pivotal triggers of the angiogenic switch during tumor growth. Targeted elimination of TEMs by means of a suicide gene impaired neovascularization of human gliomas grafted in the mouse brain and induced substantial tumor regression (De Palma et al., 2005). Because TEM elimination did not affect recruitment of TAMs to necrotic tumor areas, it is unlikely that TEMs comprise precursors of other TAM populations. Rather, it would appear that TEMs represent a distinct monocyte/macrophage subset with inherent proangiogenic activity; indeed, TEMs possess proangiogenic activity already when they circulate in the peripheral blood, before reaching the tumor site. In this regard, identification of proangiogenic TEMs among the heterogeneous TAM population may challenge the notion that transition of tumor macrophage phenotype between growth-inhibitory and growth-promoting activity is exclusively and contextually modulated by the tumor microenvironment (Balkwill et al., 2005; De Palma et al., 2005).

Neutrophils

The role of neutrophils in tumor progression has been controversial. During inflammatory responses, neutrophils are among the first cells to arrive at inflamed sites, where they phagocytize cellular debris and microorganisms and release chemokines and proteases that in turn recruit additional immune effector cells. Moreover, neutrophils are involved in

graft rejection, indicating that they might also be tumoricidal. However, whereas some reports have shown that increased neutrophil infiltration was linked to poor outcome, others suggested that neutrophil infiltration correlated with favorable prognosis (Lin and Pollard, 2004). Despite contradictory clinical studies, experimental studies that using mouse tumor models have found that tumor-associated neutrophils are involved in tumor angiogenesis and therefore can be pro-tumoral. The proangiogenic activity of neutrophils may derive from their production of canonical proangiogenic factors, such as VEGF, IL-8, MMPs and elastases (Benelli et al., 2003). Recently, Hanahan and colleagues investigated the putative role of neutrophils in multi-stage pancreatic carcinigenesis (Nozawa et al., 2006). These authors observed that scarce MMP-9-expressing neutrophils were located inside angiogenic islet dysplasias and tumors, whereas more abundant MMP-9-expressing macrophages were mainly distributed along the periphery of such lesions. Interestingly, the transient ablation of neutrophils by using anti-Gr-1 antibodies reduced the frequency of angiogenic switching in pre-neoplastic lesions, but did not inhibit angiogenesis and tumor progression in late-stage tumors. These data are consistent with the proposition that neutrophils, that are 10-fold less abundant than macrophages in both angiogenic islets and tumors, may provide an initial, non-redundant source of MMP-9 for catalyzing the angiogenic switch in pre-angiogenic lesions.

Dendritic Cell Precursors, Myeloid Suppressor Cells and Endothelial-like Monocyte-derived Cells

Dendritic cells (DCs) play important roles both in activation and suppression of anti-tumor immunity. Recently, DC precursors have been implicated in tumor angiogenesis (Conejo-Garcia et al., 2005; Coukos et al., 2005). Coukos and colleagues described a population of $CD45^+CD11c^+MHC\text{-}II^+$ DC precursors that infiltrated human ovarian carcinomas. These cells were termed "vascular leukocytes" because, in addition to the aforementioned hematopoietic markers, they expressed EC-specific markers, including vascular endothelial cadherin (VE-Cad). $CD45^+VE\text{-}Cad^+$ cells isolated from human ovarian cancers by cell sorting formed perfused vascular channels in matrigel *in vivo*, suggesting that vascular leukocytes can directly participate in angiogenesis. The mixed hematopoietic/EC phenotype of vascular leukocytes is consistent with other reports demonstrating that monocytes and immature myeloid cells can acquire an endothelial-like phenotype under angiogenic conditions (Schmeisser et al., 2001; Urbich et al., 2003).

Another myeloid population recently implicated in tumor angiogenesis are the so-called ~~immature~~ myeloid suppressor cells (Gallina et al., 2006; Serafini et al., 2006). Myeloid suppressor cells express low to undetectable levels of MHC-II and co-stimulatory molecules, therefore they cannot induce anti-tumor responses. Rather, these cells promote tumor development by exerting a profound inhibitory activity on both tumor-specific and non-specific T lymphocytes and, as recently described, by providing factors essential for tumor growth and neovascularization (Yang et al., 2004). The frequency of myeloid suppressor cells is significantly increased in the BM and spleen of cancer patients and mice carrying large tumors. Lin and colleagues (Yang et al., 2004) found that $Gr\text{-}1^+CD11b^+$ myeloid suppressor cells obtained from spleens of tumor-bearing mice promoted angiogenesis and tumor growth when co-injected with tumor cells. Myeloid suppressor cells produced high levels of MMP-9, and deletion of MMP-9 in these cells completely abolished their tumor-promoting activity. Similar to DC precursors described above, $Gr\text{-}1^+CD11b^+$ cells were also found to occasionally incorporate into tumor endothelium as endothelial-like cells (Yang et al., 2004).

The aforementioned studies highlight the ability of myeloid-lineage cells to promote tumor angiogenesis and sustain tumor progression. Although it appears that the proangiogenic function of myeloid cells in tumors mostly consists of the production of growth factors and matrix-remodeling proteins that stimulate angiogenic processes in a paracrine manner, the occasional luminal incorporation of myeloid cells in vascular endothelium has also been documented as a rare phenomenon often regarded as evidence for post-natal vasculogenesis (Coukos et al., 2005; Yang et al., 2004).

Monocytes/macrophages and ECs share phenotypical and functional features, including the expression of common metabolic and surface markers, as well as an ability to form vascular-like structures (Schmeisser et al., 2001). Surface markers co-expressed by ECs and hematopoietic subsets include VEGFR-1, Sca-1, Tie2, AC133, CD31 (PECAM-1), von Willebrand Factor and CD146 (S-endo-1 or P1H12). This may have led some monocyte-derived populations to be incorrectly regarded as bona fide BM-derived "endothelial progenitor cells" (Barber and Iruela-Arispe, 2006; De Palma and Naldini, 2006; Ingram et al., 2005). ~~Of note, at selected anatomical sites, ECs and macrophages associate to shape the lumina of specialised capillaries – termed sinusoids – where both cell types are thought to be in direct contact with plasma. Likewise, in certain pathological conditions, such as ischemia and cancer, monocytes can develop provisional nonthrombogenic surfaces that enable blood to rapidly circulate in hypoxic tissues (Moldovan et al., 2000; Urbich et al., 2003~~).

Monocytes are a highly plastic cell types that can modulate their phenotype according to local conditions, and increasing reports suggest that in vitro cultured monocytes can be differentiated into endothelial-like cells (Rehman et al., 2003; Schmeisser et al., 2001; Schmeisser et al., 2003; Urbich et al., 2003). These ex-vivo expanded endothelial-like cells have been shown to participate in vascular healing and angiogenesis under certain experimental conditions. This function is likely enhanced by the release of several monocyte-derived proangiogenic factors that may promote the local recruitment and proliferation of ECs (Grunewald et al., 2006; Rehman et al., 2003). ~~However, whether monocyte-derived endothelial-like cells can develop a functional behavior similar to true ECs~~

and are able to stably contribute to the vascular wall remain to be determined.

These interesting studies together indicate that monocytes/macrophages can function both as pathfinders for activated ECs and to form provisional endothelium-like structures for initial vessel formation. The ability of certain hematopoietic cells—and in particular monocytes/macrophages—to migrate within tissues, even in hypoxic conditions, and remodel the extracellular environment would account for their critical role in angiogenesis. This concept may provide an alternative explanation for the contribution of BM-derived hematopoietic cells to post-natal vasculogenesis.

Anti-inflammatory Drugs Meet Antiangiogenic Therapy

Hematopoietic cells of the innate immune system have a requisite role in tumor development, and increasing evidence suggests that one of the mechanisms by which they foster tumorigenesis is the promotion of angiogenesis (De Palma and Naldini, 2006; Ferrara and Kerbel, 2005). Inflammatory immune cells that are thought to promote angiogenesis in a paracrine manner (e.g., by releasing pro-angiogenic factors or ECM-modifying enzymes) include several myeloid cell populations. From the data discussed here, it follows that antiangiogenic therapy—such as treatments using anti-VEGF antibodies, VEGFR inhibitors or vascular disrupting agents—may be improved by drugs that concomitantly target proangiogenic inflammatory cells (Albini et al., 2005). Clinical studies have recently shown that regular use of non-steroidal anti-inflammatory drugs (NSAIDs) is associated with reduced risk of some cancers (Dannenberg and Subbaramaiah, 2003). Given the intimate association between inflammation and angiogenesis, NSAIDs may also function as angiopreventive molecules. On the other hand, the growing body of evidence that canonical regulators of angiogenesis, such as VEGF and SDF-1, also stimulate mobilization and recruitment of proangiogenic myeloid cells to tumors, has broadened the variety of cell types that might be concomitantly targeted by conventional antiangiogenic agents (Ferrara and Kerbel, 2005; Kerbel, 2006). However, assessing the specific contribution of different BM-derived hematopoietic cell types to tumor angiogenesis, together with the identification of selective targets, may have important implications for the design of improved anti-cancer therapies. Macrophages and other innate immune cells are genetically stable cells that are less likely to develop drug resistance than cancer cells, thus drugs that inhibit selected macrophage functions should hold promise for effective anticancer therapies (Condeelis and Pollard, 2006; Giraudo et al., 2004). The outstanding challenge is, however, to understand which targets, if any, could distinguish immune cells implicated in tumor growth from those that regulate immunity and tissue homeostasis.

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Chapter 1

Delineating Protease Functions During Cancer Development

Nesrine I. Affara, Pauline Andreu, and Lisa M. Coussens

Summary

Much progress has been made in understanding how matrix remodeling proteases, including metalloproteinases, serine proteases, and cysteine cathepsins, functionally contribute to cancer development. In addition to modulating extracellular matrix metabolism, proteases provide a significant protumor advantage to developing neoplasms through their ability to modulate bioavailability of growth and proangiogenic factors, regulation of bioactive chemokines and cytokines, and processing of cell–cell and cell–matrix adhesion molecules. Although some proteases directly regulate these events, it is now evident that some proteases indirectly contribute to cancer development by regulating posttranslational activation of latent zymogens that then directly impart regulatory information. Thus, many proteases act in a cascade-like manner and exert their functionality as part of a proteolytic pathway rather than simply functioning individually. Delineating the cascade of enzymatic activities contributing to overall proteolysis during carcinogenesis may identify rate-limiting steps or pathways that can be targeted with anti-cancer therapeutics. This chapter highlights recent insights into the complexity of roles played by pericellular and intracellular proteases by examining mechanistic studies as well as the roles of individual protease gene functions in various organ-specific mouse models of cancer development, with an emphasis on intersecting proteolytic activities that amplify programming of tissues to foster neoplastic development.

Key words: ADAMs, Angiogenesis, Cysteine cathepsins, Cancer, ECM remodeling, Inflammation, Metalloproteinases, Mouse models, Plasminogen activators, Proteases, Proteolytic cascades, Serine proteases.

1. Introduction

A unifying concept of cancer development is the acquisition of genetic alterations in critical genes, including oncogenes and tumor suppressor genes that provide a survival and/or proliferative advantage to mitotically active cells. However, it is now

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clear that genetically-altered neoplastic cells coopt important physiologic host-response processes early during cancer development to favor their own survival, including extracellular matrix (ECM) remodeling, angiogenesis, and activation/recruitment of innate and adaptive leukocytes (inflammation). In particular, cancer development requires reciprocal interactions between genetically altered neoplastic cells and activated ~~diploid~~ stromal cells, as well as the dynamic microenvironment in which they both live *(1, 2)*. Of note, proteases derived from activated host stromal cells have recently been identified as critical cofactors for cancer development. Although it was initially believed that matrix remodeling proteases merely regulated migration and/or invasion of neoplastic cells into ectopic tissues, there is growing evidence that proteases contribute to cancer development by regulating bioactivity of a myriad of growth factors, chemokines, soluble and insoluble matrix molecules that regulate activation and/or maintenance of overall tissue homeostasis, as well inflammatory and angiogenic programs (**Fig. 1**) *(3)*.

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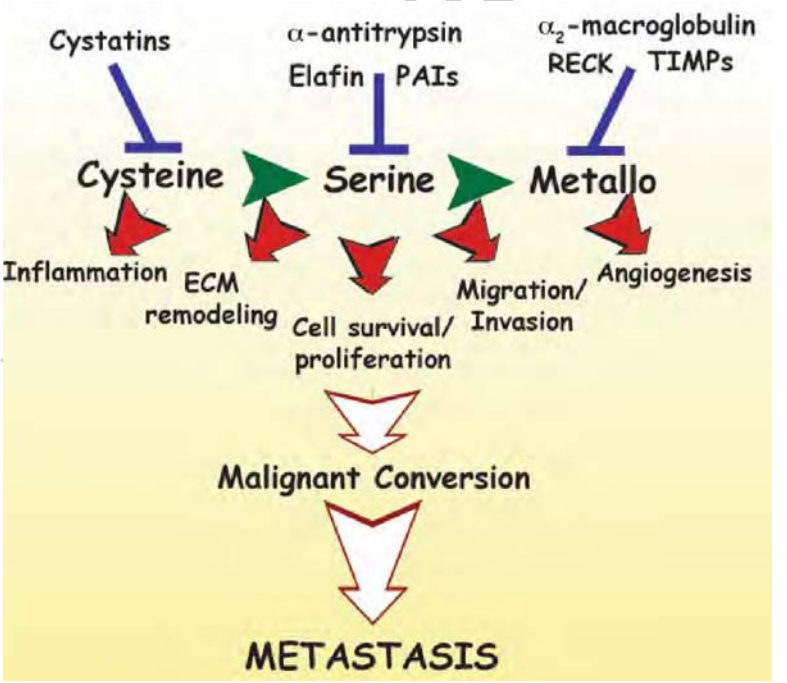

Fig. 1. Proteases act as critical cofactors for cancer development. Proteolysis, a central cofactor for neoplastic progression, results from a cascade-like activation of linear protease circuits, including key upstream proteases, such as cysteine and serine proteases, which converge leading to amplification of metalloproteinase proteolytic activities. Tumor net proteolysis contributes to tumor progression by mediating tissue remodeling, inflammation, angiogenesis, and acquisition of invasive capabilities, cell survival, and proliferation.

of proteolytic activities in cells and tissues *(9)*. Together with observations gained from examining individual protease gene functions in mouse models of de novo carcinogenesis, have emerged insights into the multitude of enzymatic activities that participate in tissue remodeling associated with cancer development *(10)*. Recently, sequencing of the human genome enabled characterization of the human degradome found to consist of at least 569 proteases and homologues that belong to various classes, including metalloproteinases (MMP), serine proteases, and cysteine cathepsins *(10)*. For many of these enzymes, their most significant protumor activity may lie in their ability to post-translationally regulate downstream proteases initially secreted as either inactive zymogens or sequestered by matrix in latent forms *(10)*. This realization has led to the notion that embedded within tissues are complex, interconnecting protease networks that, depending on the tissue perturbation, selectively engage specific protease amplification circuits *(11)*. These coordinated efforts regulate overall tissue homeostasis, response to acute damage and subsequent tissue repair, as well as contribute to pathogenesis of chronic disease states such as cancer.

2. Proteases Implicated in Cancer Development

Requisite for neoplastic cell, vascular, or inflammatory cell invasion during tumorigenic processes are the remodeling events that are initiated within tumor stroma in pericellular microenvironments. In epithelial tumors, a majority of ECM-remodeling proteases emanate from activated stromal cells, a large percentage of which being infiltrating leukocytes such as mast cells, other myeloid-lineage cells and lymphocytes *(3)*. In vivo assessment of individual protease gene functions have indeed identified some proteases as significant cofactors for cancer development because of their ability to directly regulate important aspects of neoplastic progression (**Fig. 1**), while others are significant as they set in motion interconnecting protease cascades, resulting in amplification of enzymatic activity of "terminal" proteases (**Fig. 2**). Although these cascades of proteolytic activation are crucial for tumorigenesis and resemble those regulating coagulation *(12)* and/or complement *(13)*, in vivo experimental studies ~~in~~ mouse models have revealed organ and tumor type-specific regulation of protease bioactivities, as well as involvement of proteases emanating from multiple enzymatic classes, i.e., cysteine, serine, and metallo. In the sections that follow, we discuss the diversity of proteases whose bioactivity result in amplification of *terminal* proteases in neoplastic tissues.

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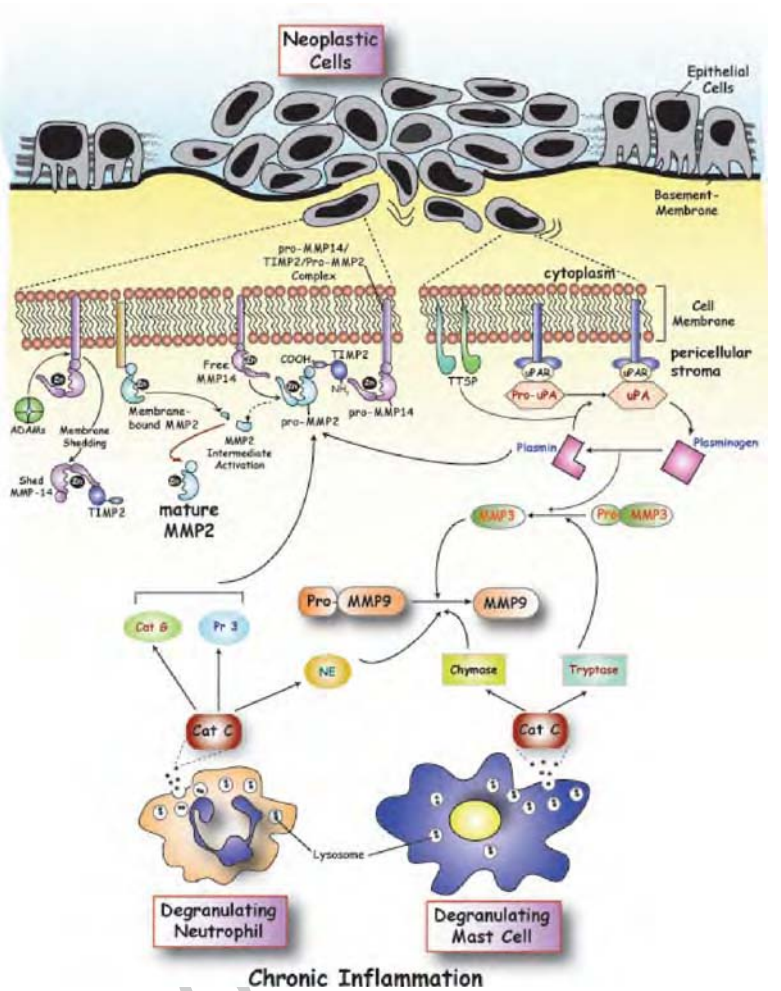

Fig. 2. Intersecting protease pathways during neoplastic progression. Proteolysis of extracellular matrix (ECM) components during tumor progression results from the activity of combined protease pathways emanating from the tumor cell compartment, including urokinase plasminogen activator (uPA), MMP14, and type II transmembrane serine proteases (TTSP), as well as proteases expressed by supporting tumor stromal cells, such as neutrophils and mast cell-derived proteases, such as MMP9, plasmin, mast cell chymase, mast cell tryptase, neutrophil elastase, and cathepsin C (Cat C). Rather than functioning individually, each protease functions as a "signaling molecule," exerting its effects as part of a proteolytic pathway, where proteases potentially interact and activate other proteases in a cascade-like manner, culminating in amplification of enzymatic activity of "terminal" proteases, such as MMP9.

2.1. Metalloprotease Activation and Function During Cancer Development

MMPs, also known as matrixins, are a family of zinc-dependent endopeptidases that act as extrinsic factors regulating critical parameters of neoplastic progression. MMPs facilitate cancer development by triggering release of growth and angiogenic factors sequestered by neoplastic tissues, as well as activation of inflammatory mediators and processing of cell–cell and cell–matrix adhesion molecules *(14)*. To date, 23 vertebrate MMPs have been identified and classified into distinct categories based

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on domain structure and substrate specificity *(14, 15)*. Bioactivity of MMP function is controlled posttranslationally. Secreted MMPs (with the exception of stromelysin-3/MMP11) remain as inactive zymogens (or pro-MMPs) requiring enzymatic and/or autolytic removal of propeptide domains, rendering the active site available to cleave substrates. However, once activated, MMPs are further regulated by three major types of endogenous inhibitors, α_2-macroglobulin, RECK *(16)*, and tissue inhibitors of metalloproteinases (TIMPs) (reviewed in *(14)*). Bioactivity of TIMPs is further regulated posttranslationally where some are inactivated by serine protease cleavage *(17)*.

The most compelling evidence for MMPs as active contributors to neoplastic progression comes from tumor-prone organ-specific mouse models harboring homozygous null gene deletions or tissue-specific overexpression of individual MMPs. In a transgenic mouse model of multistage skin carcinogenesis where the early region genes of human papillomavirus type 16 (HPV16) are expressed as transgenes under control of the human keratin 14 (K14) promoter, e.g., K14-HPV16 mice *(18)*, ~~on the one hand,~~ genetic elimination of MMP9 significantly reduced the incidence of carcinomas in K14-HPV16 mice, while on the other hand, reconstitution of K14-HPV16/MMP9^{null} mice with wild type bone marrow-derived cells restored characteristics of neoplastic development and tumor incidence to levels similar to control HPV16 mice *(19)*. Likewise, angiogenesis and tumor development were significantly inhibited during pancreatic islet carcinogenesis *(20)* and cervical carcinogenesis *(21)* following genetic deletion or pharmacological inhibition of MMP9. Similarly, during development of ovarian carcinomas using a xenograph model *(22)*, as well during neuroblastoma development *(23)*, reconstitution of MMP9-deficient mice with MMP9-proficient bone marrow-derived cells restored cellular programs necessary for development of angiogenic vasculature, tissue remodeling, and overt tumor development. Thus, in each of these distinct tissue microenvironments, infiltrating leukocytes were the predominant sources of MMP9 *(19–21)*, hence, implicating leukocyte-derived MMP9 as a significant cofactor for cancer development.

In addition to MMP9, other MMPs have also emerged as important cofactors in cancer development. Studies using genetically modified mouse models have further revealed organ and tumor type-specific regulation of MMP bioactivities. For instance, overexpression of human MMP1 (collagenase) in skin suprabasal layers not only induced epidermal hyperplasia and hyperkeratosis, but also increased susceptibility to chemical skin carcinogenesis *(24)*. But then, chemically-induced skin carcinogenesis was attenuated in MMP11 null homozygous mice *(25)*, while genetic elimination of MMP7 attenuated development of intestinal adenomas in the *m*ultiple *i*ntestinal *n*eoplasia *(Min)* mouse model of intestinal

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neoplasia *(26)*, and overexpression of either MMP2 or MMP7 in mammary epithelium accelerated mammary tumor formation *(27, 28)*. Although these studies indicate that overexpression of a single MMP may contribute to neoplastic progression *(24, 25, 28)*, MMP3 (stromelysin 1) has been shown to contribute to spontaneous mammary neoplasms by acting as a tumor promoter in the absence of carcinogens or preexisting mutations following targeting to mammary glands *(27)*. Furthermore, although mice deficient in the transmembrane-spanning MMP (MMP14/MT1-MMP) have not been specifically examined using de novo models of cancer development, the role of MMP14 as a cancer cofactor has partially been revealed using mammary-targeted MMP14 transgenic mice that developed spontaneous mammary lesions *(29)*. Nonetheless, besides promoting tumor progression, MMPs can also exhibit anti-tumor functions. For example, using MMP3-deficient mice revealed a protective role for MMP3 during chemically induced squamous cell carcinoma development *(30)*. Similarly, loss of MMP8 (collagenase 2) enhanced rather than reduced skin tumor susceptibility in MMP8-deficient male mice *(31)*.

MMP net pericellular proteolytic activity is also dependent on the balance between levels of secreted MMPs and their endogenous inhibitors, tissue inhibitors of metalloproteinases (TIMPs). Hence, elevated TIMP levels would be expected to inhibit cancer progression. Nonetheless, the contribution of TIMPs to tumorigenesis has been controversial. In contrast to overexpression of TIMP-1 that inhibited development of SV40 large T antigen-mediated liver carcinogenesis *(32)* as well as chemically-induced mammary tumorigenesis *(33)*, overexpression of TIMP-1 enhanced rather than suppressed skin carcinogenesis by promoting keratinocyte hyperproliferation and acquisition of chromosome instability, thus enhanced premalignant cells susceptibility to undergo malignant conversion *(34)*. Interestingly, once skin tumors develop, TIMP-1 acts by stabilizing tumor stroma without limiting malignant conversion and development of metastases *(34)*. Furthermore, recent studies implicated TIMP-1 in inducing a prometastatic microenvironment that promotes tumor cell metastasis selectively to the liver by triggering hepatocyte growth factor (HGF/scatter factor) signaling *(35)*. Moreover, TIMP-2 favors tumor development by acting as an adaptor molecule that induces the formation of a plasma membrane-associated ternary complex with pro-MMP2 and MMP14 *(36–38)*, thereby promoting proMMP2 activation by MMP14 at the cell surface and favoring tumor progression. Several advantages to having degradative enzymes in a bound state at the cell surface have been proposed. Namely, bound proenzymes may be more readily activated, and the bound enzymes generated may be more active than the same enzymes found in the soluble phase. Bound enzymes may also be protected from inactivation by inhibitors,

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in addition to providing a mean of concentrating components of a multistep pathway, thereby increasing rate of reactions. Immobilizing enzymes on the cell surface or in matrix may further restrict the activity of an enzyme so that substrates only in the vicinity of the cell or adjacent matrix components are degraded. Hence, activation at the cell surface may actually provide the most significant control point in MMP activity, linking MMP expression with proteolysis and invasion.

Several mechanistic studies have supported the functional contribution of MMPs to neoplastic progression. MMPs alter the stromal microenvironment by mediating liberation of ECM sequestered growth-promoting factors, such as basic fibroblast growth factor (FGF-2), or proteolytic cleavage of growth factor latent precursors, including members of the epidermal growth factor (EGF) family such as transforming growth factor-α (TGF-α), which act as potent mitogens for neoplastic cells (reviewed in *(39)*).

MMPs have also been found to act as important positive regulators of tumor angiogenesis. Infiltration of MMP9-expressing inflammatory cells coincided with development of angiogenic vasculature in premalignant skin of K14-HPV16 transgenic mice *(19)*. Although vascular endothelial growth factor (VEGF) was constitutively expressed in normal β-cells and at all stages of islet carcinogenesis, it only became bioavailable for interaction with its receptor on microvascular endothelial cells following infiltration of leukocytes expressing MMP9, thereby triggering activation of angiogenic programs *(20)*. During development of experimental neuroblastomas, MMP9 not only regulates bioavailability of VEGF, but also mediates pericyte recruitment to developing angiogenic vessels, thus inducing stabilization of newly formed tumor vasculature *(40, 41)*. An additional line of evidence supporting a role for MMP9 in promoting neovascularization comes from studies reporting the unique ability of MMP9 to induce release of soluble kit-ligand, which thereby initiated mobilization of hematopoietic stem cells/progenitor cells in bone marrow *(42,* Jodele, 2005 #12851*)*.

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Furthermore, MMP9 induces a tissue microenvironment that is permissive for primary tumor development, and its bioactivities also regulate secondary metastasis formation. Studies by Matrisian and colleagues have determined that MMP9 derived from inflammatory cells (possibly neutrophils) present in premetastatic lung facilitates survival/establishment of early metastatic cells, but not growth of metastatic foci *(43)*, whereas MMP9 derived from Kupfer cells in liver parenchyma facilitates early establishment and growth of colorectal metastases to liver. Additional clues into the later events regulating metastasis have implicated MMP9 secreted by macrophages and alveolar VEGF receptor $(VEGFR1)^{+}$-endothelial cells in microenvironmental

remodeling that is crucial for metastatic cell survival in the lung *(44)*. Using a mouse model of experimental metastasis formation, Hiratsuka et al. ~~(2002)~~ reported that following recruitment to sites of primary tumor growth, macrophages circulate to distal organs. On the one hand, distal organs exhibiting low-level expression of VEGFR1 fail to induce MMP9 in response to leukocyte presence and are therefore not suitable environments for subsequent metastatic cell growth. On the other hand, distal organs that are VEGFR1-positive and contain a population of endothelial cells capable of inducing expression of MMP9 above that supplied by circulating macrophages are "fertile" sites for productive metastatic growth. Although induced expression of the VEGFR1 ligand VEGF-A does not appear to be involved, presence of an active VEGFR1 tyrosine kinase domain is necessary; thus, it seems reasonable that activated MMP9 releases matrix-sequestered VEGF-A rendering it bioavailable for interaction with its receptors as has been reported by Bergers and colleagues *(20)*, thus stimulating efficient vascular remodeling and angiogenesis necessary for metastatic cell growth and survival. Taken together these findings indicate that mechanisms by which premetastatic niches enhance metastatic outgrowth are organ and cancer type specific.

MMPs have also been found to enhance tumor cell migration by altering cell–cell and cell–matrix interactions. For instance, MMP14 and MMP2 have been shown to release cryptic fragments of laminin-5 γ2 chain domain III, which, due to presence of EGF-like repeats, binds to EGF-receptor on tumor cells, thus activating downstream signaling events that lead to tumor cell motility *(45, 46)*. The cell–cell adhesion junction E-cadherin has also been found to act as a substrate for MMP3 in mammary epithelial cells, triggering progressive phenotypic conversion of normal epithelial cells into invasive mesenchymal phenotype, characterized by dissolution of stable cell–cell contacts, down-regulation of cytokeratins, and induction of vimentin expression *(47)*.

It is not surprising that MMPs have attracted significant attention as anti-cancer therapeutic targets. Unfortunately, clinical evaluation of MMP inhibitors revealed no efficacy in patients suffering from the advanced stages of various types of cancer *(48)*. Nonetheless, these failed clinical experiments have enabled revisiting of the upstream regulatory mechanisms controlling activation of important proteases, like MMP9, that play a clear and undisputed role in cancer development. Active MMP9 represents a terminal protease whose proteolytic activity is amplified by several proteolytic pathways that converge or act in parallel to activate the latent pro-form of the enzyme (**Fig. 2**). Indeed, serine proteinases, such as plasmin or urokinase-type plasminogen activator (uPA), neutrophil elastase, mast cell chymase, and trypsin, cleave propeptide domains of secreted pro-MMPs, such

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as MMP9, and consequently induce autocatalytic activation of MMPs *(14)*. Some activated MMPs can further activate other pro-MMPs. For example, MMP3 activates pro-MMP1 and pro-MMP9, whereas pro-MMP2 is resistant *(14)*. Thus, some serine proteinases act as initiators of activation cascades regulating bioactivity of pro-MMPs in vivo. Collectively, these observations indicate that anti protease-based therapeutics may achieve better efficacy when targeting a "pathway" as opposed to a single class or single species of enzyme(s).

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2.2. ADAMs: Emerging Roles in Tumorigenesis

ADAMs (A disintegrin and metalloproteinase domains) are a family of cell surface proteins related to snake venom metalloproteases (SVMPs) and MMPs, characterized by the presence of both a desintegrin and metalloproteinase domains responsible for their wide range of biological activities, including proteolysis, adhesion, and signaling *(49)*. More than 30 ADAM orthologues have been identified in various species, in particular 29 are found in mammals, of which several members are expressed exclusively in the testis where they participate in spermatogenesis and fertilization. ADAMs are typically composed of the following structural and functional conserved domains: a prodomain acting as an intramolecular chaperone responsible for the protease domain inhibition, a metalloprotease domain, a desintegrin domain shown to interact with integrins to regulate cell–cell adhesion, a cysteine-rich region implicated in both cell–cell and cell–ECM interactions because of its abilities to link heparan sulfate proteoglycan, an EGF repeat domain, a transmembrane, and a cytoplasmic domain *(49–51)*. Despite the presence of a metalloprotease domain, only 17 of the 29 mammalians ADAMs identified at this date contain the catalytic consensus motif for metalloproteinases, indicating that half of the ADAMs have actual catalytic activities that have been formally proven to exist for ADAMs 10, 12, 17, and 28 *(51–55)*.

ADAMs expression has been found to be induced in human cancers, particularly ADAMs 9, 10, 12, 15, and 17, which are expressed at low levels in normal tissue but overexpressed in a variety of tumors, including breast, gastric, colon, prostate and pancreas carcinomas, nonsmall cell lung cancer, liver metastases, glioblastomas, as well as hematological malignancies *(56–63)*. Moreover, studies have demonstrated a predictive role of ADAMs expression for human breast cancer, as ADAM17 expression levels predict poor prognosis *(64)* and urinary concentration of ADAM12 correlates with cancer progression *(60)*.

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Unlike other proteases families, a role for ADAMs in tumorigenesis has only recently begun to be explored using a growing number of de novo carcinogenesis mouse models harboring either gene deletion or tissue-specific overexpression of individual ADAM. Using the W^{10} mouse model of prostate cancer where

SV40 large T antigen is expressed under the control of the probasin promoter, homozygous deletion of ADAM9 inhibited cancer progression past the well-differentiated stage *(59)*. Conversely, transgenic overexpression of ADAM9 in mouse prostate epithelium was sufficient to induce epithelial hyperplasia leading to the development of neoplastic lesions after 1 year *(59)*, thus revealing a functional role for epithelial-derived ADAM9 in prostate cancer initiation and progression likely related to ability of ADAM9 to shed FGF receptor (FGFR2iiib) and EGF both implicated in human prostate cancer development *(65, 66)*. In the same W^{10} mouse model, ADAM12 expression was restricted to a subpopulation of stromal cells where it exerts an essential role in tumor progression and development as genetic deletion of ADAM12 resulted in smaller and more differentiated neoplastic lesions *(67)*. Accordingly, driven by the mammary epithelium promoter MMTV, overexpression of either the secreted form of ADAM12 or a membrane-anchored form lacking the intracellular domain increased both tumor burden and malignancy degree of breast carcinomas in MMTV-PyMT mice *(68)*. These observations indicate that ADAM12 extracellular region, including the protease and adhesion domains, mediates ADAM12-tumor promoting capabilities, as opposed to initiation of a signaling cascade through ADAM12 intracellular tail.

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In vitro, ADAM17 expression regulates glioma tumor cell invasiveness *(62)* and human pancreatic ductal adenocarcinoma proliferation and invasive abilities *(69)*. These effects may be related to cell migration, adhesion, or matrix remodeling activities of ADAMs. Several ADAMs have indeed been found to degrade specific components of ECM, i.e., ADAM12 mediates processing of gelatin, type IV collagen, and fibronectin *(60)*, ADAMs 10 and 15 induce type IV collagen and gelatin degradation *(70, 71)*, and ADAM13 has been reported to cleave fibronectin *(72)*. Thus ADAMs-mediated cleavage of ECM proteins not only fosters cancer cell migration but also induces release of ECM-sequestered growth and angiogenic factors.

Although direct evidence is still lacking, ADAMs are also suspected to exert a functional role in tumor angiogenesis. In a mouse model of retinopathy of prematurity, mice harboring a homozygous deletion of ADAM15 present an inhibition of angiogenesis when compared with the control mice, implicating ADAM15 in the control of pathogenic angiogenesis. However, although growth of implanted tumors has also been inhibited in $ADAM15^{-/-}$ mice, there was no difference in tumor vascularity *(73)*. Moreover ADAM15 and 17 are expressed in endothelial cells (EC) *(74)* where they may exert an important functional role, since using an ADAM-specific inhibitor (GLI12971) decreased EC migration, adhesion, and proliferation in vitro *(75)*.

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One mechanism by which active ADAMs functionally contribute to neoplastic progression is through their sheddase activity, defined as the proteolytic release of ectodomains of membrane-anchored cell-surface proteins. The sheddase activity of ADAMs regulates numerous signaling proteins, including growth factors and receptors, whose activation and/or bioavailability are dependant of several proteolytic steps. Importantly in cancer, ADAMs have been implicated in the shedding of EGFR ligands, including TGFα, EGF, HB-EGF, betacellulin, epiregulin, and amphiregulin (reviewed in *(49)*), implicating them as a major regulator of EGFR signaling. The signals initiated by EGFR-like receptors have been extensively studied since the early 1980s and demonstrated to participate in the control of differentiation, proliferation, and cell survival as well as in the development of tumors from epithelial origin *(76)*. In particular, expression of ADAM17, initially termed tumor necrosis factor-α converting enzyme (TACE), is specifically induced in breast and nonsmall cell lung carcinomas (NSCL) where it seems necessary for EGFR signaling *(63, 77)*. In 3D culture model of human breast cancer progression, inhibition of TACE/ADAM17 by siRNA or small molecule inhibitors reduces breast cancer cell proliferation and reverts their malignant phenotype by inhibiting EGFR signaling *(64)*. Similarly, use of a selective ADAM inhibitor in NSCL cell lines contributes to the inhibition of EGFR signaling *(63)*. Several anti-EGFR agents have been approved to treat various human cancers, but despite their efficiency, the majority of patients do not experience long-term benefit from these therapies *(78–80)*, demonstrating a need for alternative strategies to target EGFR signaling pathway. As such, ADAMs inhibitors may offer a new opportunity for pharmacological interventions by targeting upstream the receptor that could be then used in combination with others drugs.

Furthermore, because of their ability to regulate levels of chemokines and cytokines, including tumor necrosis factor-α (TNF-α), TRANCE, CX3CL-1, CXCL-16 *(81)*, ADAMs have also been implicated in the recruitment of immune cells implicated in inflammatory. For instance, TNF-α is synthesized as a transmembrane precursor that is processed through the proteolytic activity of ADAM17/TACE to a soluble form. Interestingly, mice lacking TIMP-3 develop *inflamed* livers associated with an increase in TNF-α activity *(82)*. The mechanism includes ability of TIMP-3 to inhibit ADAM17; thus, TIMP-3 regulates ectodomain shedding of TNF-α under physiological conditions, which if perturbed, results in high levels of soluble TNF-α and development of spontaneous inflammation.

Another intriguing role for ADAMs in tumorigenesis was proposed recently, namely shedding of membrane-anchored

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MMPs, including MMP14 *(83)*. As such, shed MMP14 may compete with membrane-anchored MMP14 for TIMPs, thus altering the balance of active MMPs and their inhibitors at the cell surface as well as the vicinity of tumor cells (**Fig. 2**) *(84)*. Collectively, recent observations presented here implicate ADAMs as cofactors for cancer development. These observations shed light on the importance of understanding how a tissue responds under homeostatic circumstances by differentially activating specific protease pathways, when compared with that response following pathologic challenge. Thus, although it is evident that several protease pathways may converge, the above-mentioned studies indicate existence of protease pathways that may act in parallel to foster neoplastic progression.

2.3. Serine Protease Regulation of MMP Activity During Cancer Development

Several serine proteases have been implicated as important regulators of cancer development, some of which are known regulators of MMP9 bioactivity. This protease family includes enzymes involved in mediating activation of plasminogen (urokinase-type and tissue-type plasminogen activators, uPA and tPA, respectively) *(85)*, as well as serine proteases stored in secretory lysosomes of various leukocytes, namely mast cell chymase *(86)*, mast cell tryptase *(86)*, and neutrophil elastase (NE) *(87)*. Although most secreted serine proteases emanate from host stromal cells, recent studies implicate a superfamily of cell-surface associated serine proteases, also known as Type II transmembrane serine proteases (TTSP), such as matriptase/MT-SP1 and hepsin, originating exclusively from tumor cells as important regulators of cancer development *(88)*.

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Serine proteases share a common characteristic that being their synthesis as inactive zymogens whose activation involves a two-step mechanism *(89, 90)*. Following synthesis and signal peptide removal during passage through the endoplasmic reticulum, a pro-protease containing an "activation dipeptide" is generated, characterized by the presence of two amino-terminal residues that blocks substrate access to the catalytic site cleft, thereby maintaining serine proteases in their latent state and preventing premature protease activation. Furthermore, serine protease proteolytic activity is tightly regulated by acidic pH, an environment that is typical of secretory granules.

2.3.1. Plasminogen Activators

Several mechanistic studies have implicated the uPA/plasmin proteolytic cascade in functionally contributing to neoplastic progression, including acquisition of a migratory and invasive phenotype by tumor cells, as well as remodeling of ECM components via activation of a number of MMPs, such as MMP9 *(91)*. Enzymatic activity of plasmin is tightly regulated by two plasminogen activators, uPA and tPA. uPA plays a crucial role in tissue remodeling, while tPA is important in vascular fibrinolysis.

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Initiation of plasmin activation occurs following binding of uPA to its receptor uPAR, a gycosyl-phosphathidylinosityl (GPI)-anchored cell membrane protein found to localize to discrete focal contacts *(92)*. Subsequently, ~~on the one hand,~~ binding of uPA to uPAR catalyzes conversion of plasminogen to its active form, plasmin. On the other hand, uPAR-bound pro-uPA is also activated by plasmin, in turn results in a feedback pathway that accelerates plasminogen activation *(93)*. Maintenance of this protease cascade depends upon the balance between uPAR-bound uPA proteolytic activity and endogenous inhibitors, plasminogen activator inhibitor-1 (PAI-1) *(94)*. PAI-1 not only regulates proteolytic activity of uPA, but also induces rapid internalization of the uPA/PAI-1/uPAR complex *(95)*, thus regulating levels of cell surface-bound uPA as well.

Despite extensive experimental data linking uPA/uPAR system to neoplastic progression, only few studies have evaluated the role of the uPA proteolytic pathway in tumor-prone organ-specific mouse models *(96, 97)*. Although the number of intestinal adenomas in the *Min* mouse model of intestinal neoplasia ($Apc^{Min/+}$) was significantly reduced following genetic elimination of uPA, proliferation and angiogenesis of established neoplastic lesions were not altered in the absence of uPA, most likely due to mechanisms involving up-regulation of cycloxygenase-2 (Cox-2) expression and the Akt signaling pathway *(96)*. These observations implicate a tumor promoting role of uPA during the early stages of intestinal adenoma development through mechanisms involving leukocyte infiltration *(96)*.

Further mechanistic studies implicated uPA/plasmin proteolytic cascade as a key event conferring tumor cells with ability to migrate through fibrinous matrices. By directly activating pro-MMPs, including pro-MMP-1, -2, -3, and -9 *(91, 98–100)*, plasmin contributes to localized extracellular proteolysis and ECM remodeling at the leading edge of migrating tumor cells. These data support the presence of a cascade of proteolytic activations that converge leading to activation of common terminal proteases, including MMP9 (**Fig. 2**).

2.3.2. Mast Cell Serine Proteases

The functional significance of mast cell-derived serine protease activity (chymases and tryptases) in neoplastic progression has recently been appreciated, given their ability to trigger a proinflammatory response as well as to induce a cascade of protease activation, culminating in activation of MMP9 (**Fig. 2**). While mast cell-derived chymases and tryptases are stored in secretory granules, their release into the extracellular milieu is triggered following activation/degranulation in response to cross-linking of FcεRIe, a high affinity receptor for immunoglobulin E (IgE) *(101)*. Mast cell activation can also occur independently of IgE via mechanisms involving the complement system (C3a and C5a) *(102)*,

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neuropeptides such as substance P *(103)*, cytokines including stem cell factor *(104)*, as well as engagement of the Toll-like receptors *(105)*.

Although one human chymase gene belonging to the α chymase family has been identified to date, rodents express several β chymases (murine mast cell protease/mMCP-1, -2, and -4) in addition to α chymase (mMCP-5) *(106)*. Notably, chymotrypsin-like activities in murine peritoneal cells and cutaneous tissue (skin) were absent following genetic elimination of mMCP-4 *(107)*, indicating that mMCP-4 is the major source of stored chymotrypsin-like activity in these tissues. On the other hand, mast cell tryptases in rodents include mMCP-6 and mMCP-7. Following degranulation, mMCP-6 is secreted locally into the vicinity of mast cells, while mMCP-7 is released into the circulation *(108)*. A novel mast cell tryptase, denoted mMCP-11/mastin, has recently been identified in dogs, pigs, and mice *(109)*. In humans, tryptases include membrane-anchored tryptases (γ-tryptase) and soluble tryptases (α-, β-, and δ-tryptases), the latter that has also been termed mMCP-7-like tryptases because of similarities with mMCP-7 genetic organization. However, β-tryptases represent the most important tryptases following secretion, and α- and δ-tryptases are both resistant to activation because of the presence of propeptide mutations and catalytic domain defects (reviewed in *(110)*). Tryptases are known to cleave substrates at the carboxyl-terminal side of basic amino acids, while chymases exert chymotrypsin-like activity, cleaving peptides at the carboxyl-terminal side of aromatic amino acids *(111, 112)*.

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Although specific gene knockouts of murine chymases and/or tryptases have yet to be evaluated in de novo mouse tumor models, their role in cancer appears clear since mechanisms delineating their functional contribution to neoplastic progression have recently been examined. For instance, injection of human chymase into skin of guinea pigs induced a significant increase in neutrophil infiltration and vascular permeability *(113)*, indicating the ability of mast cell chymase to exert proinflammatory effects. Furthermore, although pro-MMP9 represents a common terminal substrate that is activated by many upstream proteases, mast cell-derived mMCP-4 plays a crucial role in MMP9 activation, given that only the proform of MMP9 was detected in tissue extracts in the absence of mMCP-4 *(107)*. α-chymase has also been shown to indirectly mediate MMP9 activation by catalyzing cleavage and hence inactivation of free TIMP-1, as well as TIMP-1 when bound in a complex with pro-MMP9 *(17)*. Remarkably, through its ability to activate MMP9, chymase exerts indirect proangiogenic activities via regulating release of sequestered VEGF from the matrix *(86)*. Moreover, a major role of mast cell-derived chymase in regulating turnover of connective tissue components, including thrombin, fibronectin, and

collagen has been revealed by evaluating skin, heart, and lung tissues in mMCP-4 homozygous null mice *(107)*. In addition to activating MMP9, studies using mMCP-4-deficient mice indicate that mMCP-4 is important but not essential for activation of pro-MMP2 in vivo, since reduced levels of active MMP2 have been observed in the absence of mMCP-4 when compared with wild type mice *(107)*.

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Mast cell tryptases have been implicated in cancer development because of their ability to activate MMP9 indirectly by initially inducing pro-MMP3 activation *(114)*. Furthermore, mast cell-derived tryptases modulate neoplastic microenvironments during skin carcinogenesis by acting as direct mitogens for stromal fibroblasts *(115, 116)* and epithelial cells *(117)*, in addition to stimulating synthesis of α_1 pro-collagen mRNA in dermal fibroblasts *(86)*. Tryptases also act as potent proinflammatory factors, given that injection of mMCP-6, but not mMCP-7, induced neutrophil infiltration into peritoneal cavities *(118)*. Furthermore, recent studies suggest that tryptases indirectly induce leukocyte recruitment by stimulating chemokine release, such as IL-8, from endothelial and epithelial cells *(117, 119)*.

The above-mentioned findings reveal that mast cell-derived chymases and tryptases are both functionally capable of modulating the tumor microenvironment by mediating dissolution of ECM components and triggering inflammation. Significantly, chymases and tryptases are stored in mast cell secretory granules in their mature and enzymatically active forms, ready for exocytic release following mast cell degranulation, as opposed to most MMPs that are secreted as zymogens requiring pericellular activation. A cysteine protease *(120)*, cathepsin C (also known as dipeptidyl peptidase I (DPPI)), particularly abundant in mast cell secretory granules, acts as a direct upstream activator of mast cell serine proteases. These observations indicate that mast cell-derived chymases and tryptases functionally connect members of the cysteine protease family with terminal proteases, such as MMP9, indicating the presence of a cascade of proteolytic activations that may serve as potential anti-cancer intervention strategies.

2.3.3. Neutrophil-Derived Serine Proteases

Neutrophil elastase (NE), a serine protease abundantly present in neutrophil azurophilic granules, is transcriptionally activated early during myeloid development *(121, 122)*. Little is known about the role of NE in cancer progression, while the role of NE in pulmonary disorders, including emphysema and fibrosis, has been well documented *(123)*. Interest in NE during neoplastic processes stems from recent clinical reports that correlate elevated NE expression with poor survival rates in patients with primary breast cancer *(124)* and nonsmall cell lung cancer *(125)* as well as having recently been found to initiate development of acute promyelocytic leukemia (APL) by mediating direct catalytic

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cleavage of PML-RARα, a protein generated by a chromosomal translocation fusing promyelocytic leukemia (*PML*) and retinoic acid receptor-α (*RARα*) genes *(126)*. Further mechanistic studies revealed the ability of NE to directly modulate ECM components or indirectly through initiation of protease cascades, culminating in activation of terminal MMPs, such as pro-MMP9 *(127)*. In addition, NE exerts proinflammatory potential by enhancing neutrophil migration into inflamed tissues *(128)*. Interestingly, NE facilitates transendothelial migration of neutrophils through its ability to localize to neutrophil plasma membrane following exocytosis *(129)*.

Although initially thought to simply mediate intracellular clearance of bacteria *(130, 131)*, recent studies extend the role of NE to a modulator of inflammatory responses. Experimentally, localization of active NE to the outer surface of plasma membrane enables neutrophil transmigration in vivo *(132)*. Using in vivo intravital microscopy and in the presence of selective NE inhibitors, neutrophil adhesion to postcapillary venules and emigration out of vasculature were attenuated *(133)*. This role is further supported by studies using a mouse model of acute experimental arthritis that found reduced neutrophil infiltration into subsynovial tissue spaces in NE-deficient mice *(134)*, which were also found to exhibit reduced incidence of ultraviolet B (UVB) and chemically (Benzopyrene)-induced skin tumors *(87)*. It remains to be established whether reduced tumor incidence occurred as a result of impaired NE-mediated cleavage of ECM substrates, deficient MMP9 activation, or diminished cleavage of NE substrates such as ECM components or E-selectin on endothelial cells *(135)*. In addition, using an air-pouch model of inflammation, neutrophil emigration to sites of inflammation was completely attenuated in response to zymosan particles following genetic elimination of both NE and cathepsin G (a cysteine protease) *(134)*. However, neutrophil recruitment to *inflamed* tissues was not altered in response to lipopolysaccharide (LPS) in the absence of NE *(136)*. Notably, although neutrophils migrated normally to sites of bacterial infection in NE-deficient mice, their ability to initiate intracellular killing of gram-negative bacteria was altered *(137)*, thus indicating some degree of specificity for recruitment and response regulated by NE.

The ability of NE to differentially regulate neutrophil recruitment in "damaged" tissues, while directly significant for acute inflammatory responses, is also significant in the context of the role of neutrophils in mediating remodeling of matrix in tissues. Indeed, neutrophil-derived proteases have been identified as important regulators of insoluble elastin *(138)*, a structural component of tissues such as blood vessels, skin, and lung, in addition to hydrolysis of other ECM components, including fibronectin *(139)*, proteoglycans, and type IV collagen catabolism *(129, 134, 140)*.

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Furthermore, NE can indirectly modulate structural components of tumor ECM by activating MMPs, thus facilitating tumor cell migration. Although soluble pro-MMP2 is resistant to activation by NE, pro-MMP2 becomes susceptible to activation by NE following binding to membrane-anchored MT1-MMP/MMP14 *(141)*. Furthermore, NE has also been shown to activate pro-MMP3 *(142)*, as well as pro-MMP9 *(127)*. MMP9 can in turn cleave the NE inhibitor α1-anti-trypsin, thereby indirectly enhancing NE enzymatic activity *(143)*.

Central to the proposed roles of NE in tumorigenesis is the mechanism of NE activation and identification of potential target substrates. Synthesized as an inactive zymogen, NE requires posttranslational removal of the amino-terminal dipeptide for enzymatic activation *(144)*. Through cathepsin C catalytic activity, cleavage of propeptide occurs prior or during transport of NE to neutrophil azurophil granules. Thus following neutrophil degranulation in response to various cytokines and chemoattractants, NE is secreted in its catalytically active form. One mechanism by which NE acquires resistance to the inhibitory effects of circulating proteinase inhibitors, including α1-anti-trypsin *(145)*, is by localization to neutrophil cell surface following fusion of primary granules with plasma membranes during exocytosis *(146)*. Catalytically active NE is rapidly released from azurophil granules following excessive neutrophil influx at sites of inflammation, along with terminal proteases including pro-MMP9 stored in its zymogen form in neutrophil gelatinase granules. In turn, catalytically active NE induces activation of secreted pro-MMP9 within tumor microenvironments. One way neutrophil-derived NE can significantly contribute to net tumor proteolysis by catalyzing activation of pro-MMP9 originating from other cell populations, including infiltrated mast cells, hence resulting in amplification of MMP9-mediated effects.

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2.3.4. Membrane-Anchored Serine Proteases: Emerging Roles in Cancer Development

Most serine proteases are expressed by supporting tumor stromal cells, such as mast cells and neutrophils, whereas membrane-anchored serine proteases, also known as Type II transmembrane serine proteases (TTSP), appear to be largely expressed by tumor cells *(147)*. Much attention has recently been focused on membrane-anchored serine proteases, such as matriptase and hepsin, given their remarkable up-regulation in human cancers of epithelial origin, including carcinomas of skin, breast, and prostate *(148)*. Moreover, shedding of TTSP extracellular domains in seminal fluids and serum of cancer patients could represent a potential marker for cancer development and recurrence *(149)*.

Mouse models of cancer development have provided insights into which protease members of this family play significant roles in tumorigenesis processes. On the one hand, overexpression of

Affara, Andreu, and Coussens

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matriptase in mouse epidermis induced spontaneous skin lesions in the absence of genetic alterations and independent of carcinogen exposure *(150)*. On the other hand, using a mouse model of nonmetastasizing prostate cancer, overexpression of hepsin resulted in primary tumor progression and metastasis to liver, lung, and bone *(151)*; however, it remains to be determined how matriptase and hepsin promote neoplastic progression in these mouse models. Anchoring of TTSP to the plasma membrane directs proteolytic activities to specific compartments of tumor cell surface, including cell/ECM contacts and the invasive fronts of migrating tumor cells, thus facilitating tumor cell invasion *(151)*. Furthermore, emerging observations indicate an interesting role of matriptase and hepsin in initiating a cascade of proteolytic activations at tumor cell surface. Of note, both matriptase and hepsin have been shown to act as potent activators of receptor-bound pro-uPA *(152, 153)*, thus amplifying plasminogen conversion to plasmin by activated pro-uPA. Significantly, matriptase may promote tumor progression by directly acting as a major activator of matrix-degrading proteases, including pro-MMP3 *(154)*. In turn, activated MMP3 catalyzes activation of other MMPs, including MMP9 and MMP1 *(14)*. Extensive investigation is required to delineate upstream activators of TTSP *(148)*. Nonetheless, it is clear that tumor-associated serine proteases, notably matriptase and hepsin, contribute significantly to tumor progression via their ability to initiate a proteolytic cascade of zymogen activation, including uPA/plasmin and MMP3, collectively culminating in activation of terminal proteases, such as MMP9.

In summary, current insights regarding protease degradomics reveal that despite targeting distinct matrix substrates, serine proteases exhibit partially overlapping target substrate profiles, such as activation of pro-MMP9, whose net activity is significantly amplified during pathological processes and following simultaneous activation of various serine protease circuits originating from different cellular compartments, such as mast cells and neutrophils. Alternatively, although these protease pathways may be individually redundant, the above observations support the notion that serine proteases may profoundly influence neoplastic progression by acting collectively.

2.4. Cysteine Cathepsin Protease Regulation in Cancer Development

Cathepsins are prototypical lysosomal cysteine proteases sharing a conserved active site cleft in which amino acid residues cysteine and histidine constitute the catalytic ion pair, a distinctive characteristic of the papain-like superfamily of cysteine proteases *(155)*. Human cathepsins comprise 11 members including cathepsins B, C, H, F, K, L, O, S, V, W, and X/Z *(156)*. Although several family members have been identified as important regulators of cancer development, our laboratory has focused on cathepsin C because of the significant role it plays in regulating multiple

serine proteases that together act in concert to mediate important immune-based aspects of cancer development, namely those culminating in the conversion of pro-MMP9, thus greatly amplifying MMP bioactivity.

In particular, many unique structural features contribute to the distinctive activities mediated by cathepsin C. Although most cathepsins act as endopeptidases, cathepsin C represents the only exception by acting as a dipeptidyl aminopeptidase, cleaving two-residue units from the N-terminus of a polypeptide chain *(157)*. Furthermore, localization of cathepsin C active site to the external surface of the protein confers cathepsin C with an advantage of hydrolyzing diverse groups of chymotrypsin-like proteases in their native state, regardless of size *(155)*, as opposed to other oligomeric proteases, including tryptases, where active site are located inside of the protein *(158)*.

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Cathepsin proteolytic activity is regulated at various levels, including posttranslational mechanisms. All members of the cathepsin family share in common the general mechanism of activation that is being synthesized as zymogens, therefore sharing the presence of a signal peptide and propeptide sequence removed at maturation *(159)*. To note, a residual portion of the propeptide, termed the exclusion domain, remains bound to the catalytic part of active cathepsin C *(160)* that contributes to formation and stabilization of the tetrameric structure of the mature enzyme *(161)*. In vitro studies exclude autocatalytic activation of procathepsin C *(162)*, but whether activation of cathepsin C is facilitated by other proteases is yet to be determined.

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Although initially thought to simply mediate terminal intracellular protein degradation within lysosomes, diverse biological functions of cathepsins have come to light through recent mechanistic studies, including interstitial thrombin and fibronectin metabolism, cytotoxic lymphocyte-mediated apoptotic clearance of virus-infected and tumor cells *(163)*, survival from sepsis *(164)*, and experimental arthritis *(144)*. Furthermore, using de novo carcinogenesis models in cathepsin-deficient tumor-prone mice has implicated specific roles for individual cathepsins in distinct tumorigenesis processes. A complex cascade of sequential cathepsin expression correlating with tumor development has been documented following profiling of expression and activity of cathepsins in normal, premalignant, and malignant islets of RIP1-Tag2 mice *(165, 166)*. Joyce and colleagues further assessed the unique roles of cathepsins during pancreatic islet carcinogenesis by genetic elimination of individual cathepsin genes. Although absence of cathepsin C was without consequence, tumor-associated angiogenesis was significantly reduced in the absence of cathepsin B or S, while genetic elimination of cathepsin B or L instead attenuated tumor cell proliferation and decreased tumor volume *(165)*. Similar studies by Peters and colleagues found that during mammary

carcinogenesis in MMTV-PymT transgenic mice *(167)*, absence of cathepsin B emanating from macrophages significantly limited primary tumor development as well as pulmonary metastasis formation *(168)*. Interestingly, although cathepsin B was found to be a significant protumor regulator of pancreatic and mammary carcinogenesis, cathepsin B does not appear to be functionally significant during skin carcinogenesis (Junankar and Coussens, unpublished observations). Moreover, absence of cathepsin C during islet carcinogenesis is without consequence, whereas its absence during squamous carcinoma development in K14-HPV16 transgenic mice is profound (Junankar and Coussens, unpublished observations). During murine skin carcinogenesis, like in humans afflicted with loss of mutations in the *cathepsin C* gene *(169–172)*, myeloid cells fail to infiltrate damaged tissue *(144, 173, 174)*; thus, protumor programs (angiogenesis, matrix remodeling) regulated by inflammation fail to be activated.

In addition to maintaining tissue homeostasis and regulating diverse enzymatic activities, recent studies implicated cathepsin C as a mediator of inflammation. Although mice harboring homozygous deletions in the *cathepsin C* gene were resistant to experimental acute arthritis and displayed altered neutrophil recruitment in response to zymosan and immune complexes, a defective response that was rescued by administration of a neutrophil chemoattractant, cathepsin C-deficient mice exhibited normal neutrophil chemotactic responses to thioglycollate *(144)*. Likewise, on the one hand, using an air pouch model of inflammation, the number of infiltrating neutrophils was significantly attenuated in mice deficient in both NE and cathepsin G ($NE^{-/-} \times CG^{-/-}$) *(144)*, which was associated with a significant decrease in local levels of chemokines, including TNF-α and interleukin-1β (IL-1β). On the other hand, injection of IL-8, a neutrophil-specific chemokine, restored infiltration of neutrophils into air pouches of cathepsin C-deficient mice *(144)*. These results provide novel insights into the functional significance of cathepsin C-dependent proteolytic cascades in regulating local levels of chemoattractants at sites of inflammation *(144)*.

It has been recently highlighted that cathepsin C fosters tumorigenesis by acting as an important upstream regulator of multiple proteolytic events, mainly by activating several serine proteases. Using cathepsin C null homozygous mice, cathepsin C has been found to be essential for intracellular activation of neutrophil-derived proteases, including NE, cathepsin G, and proteinase 3 *(144)*. Similarly, cytotoxic T lymphocyte-derived granzymes A and B were found to be inactive and present in their proforms in the absence of cathepsin C *(174)*. Of note, while cathepsin C is necessary for activation of mouse mast cell chymase (mMCP-4) *(120)*, recent studies suggest that cathepsin C may not be essential for activation of mouse mast cell tryptases (mMCP-6) *(120, 175)*.

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Indeed, in vitro studies support these observations, indicating that pro-β-tryptase undergoes auto-cleavage, resulting in a two amino acid residue activation dipeptide sequence that is subsequently catalyzed by cathepsin C *(176)*.

As mentioned earlier, cathepsin C initiates a cascade of proteolytic activation. Following activation by cathepsin C, mMCP-4, the major source of stored chymotrypsin-like activity in mouse peritoneum and skin *(106)*, further activates pro-MMP2 and pro-MMP9 *(107)*. In addition to mMCP-4, neutrophil-derived NE, cathepsin G, and proteinase 3 have also been found to regulate MMP2 activation *(141)*. Although these neutrophil-derived proteases are unable to process pro-MMP2 in its soluble form, recent studies indicate that pro-MMP2 undergoes conformation changes following binding to the membrane-tethered MMP14, rendering a cleavage site within pro-MMP2 prodomain region accessible for cleavage by neutrophil-derived serine proteases *(141)*. These observations raise an interesting point – that besides the notion that these proteases converge to activate common terminal proteases, redundancy should also be noted as a common theme amongst these proteolytic cascades.

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In summary, using various mouse models of multistage cancer revealed interconnecting protease cascades that are initiated by a common upstream protease activator, cathepsin C, and culminating in amplification of enzymatic activity of "terminal" proteases such as MMP9 (**Fig. 2**). Significantly, these studies identified individual cathepsins that play differential roles in specific cancers emanating from multiple organ sites, and thus affirming the significance of organ and tumor type-specific regulation of protease bioactivities.

3. Intersecting Proteolytic Cascades in Cancer Development: A Target for Anti-Cancer Therapies?

Collectively, a vast body of literature indicates that proteolysis is central to neoplastic progression, in particular proteolytic enzymes emanating from host stromal cells. Given the data discussed here, it follows that proteases can potentially interact and activate other proteases in a cascade-like manner, resulting in the formation of protease circuits that may interconnect, forming the so-called *protease web (177)*. This concept may provide an alternative definition for proteolysis during tumorigenesis processes: each protease can be regarded as a "signaling molecule" that exerts its effects as part of a proteolytic pathway rather than simply functioning individually (**Fig. 1**). Nonetheless, in an attempt to understand the functional roles of proteases in cancer development, it is necessary to incorporate all the elements of the protease web, including

not only proteases, but also the corresponding inhibitors, cofactors, cleaved substrates, and receptors, therefore, further viewing proteolysis as a *system*. Significantly, distinct pathological conditions may arise when perturbations occur involving key upstream proteases, e.g. cathepsin C, thus profoundly affecting a myriad of downstream effectors and ultimately altering the net proteolysis as a whole. Notably, the above-mentioned studies indicate that these linear protease circuits may converge, leading to amplification of proteolytic activity of terminal proteases like MMP9 within tissues and consequently enhancing development of pathological conditions. On the basis of these observations, and given that cathepsin C functionally contribute to neoplastic progression by acting as a key upstream proteolytic enzyme, then using a single and selective drug that targets cathepsin C proteolytic activity should impede the activation of several serine proteases, including neutrophil and mast cell-derived serine proteases, thus hold promise for effective anti-cancer therapies when compared with blocking individual serine proteases.

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However, the significance of organ and tumor type-specific regulation of protease bioactivities should not be ignored when designing future anti-cancer therapies. Thus the important challenge is to characterize the "cancer degradome" at the protease and substrate levels. Which proteases are differentially active in specific tumor types? What substrates do they activate and what interconnections and networks can they potentially form? Consistent with these propositions, Joyce and colleagues investigated the effects of inhibition of cathepsin protease activity in pancreatic islet carcinogenesis using an irreversible broad-spectrum cysteine cathepsin inhibitor, JPM-OEt. Although treatment with JPM-OEt was necessary and sufficient to attenuate pancreatic tumor growth, angiogenesis, and invasiveness, however, the frequency of tumor cell apoptosis remained unaltered *(166)*. Recently, treatment with JPM-OEt in combination with the cytotoxic chemotherapeutic agent cyclophosphamide induced pancreatic tumor cell apoptosis, resulting in a more pronounced tumor regression compared with either treatment alone *(178)*. These interesting studies shed the light on the importance of using cysteine cathepsin inhibitors as effective cancer therapeutics.

Alternatively, should future studies focus on targeting multiple protease families as opposed to individual proteases? Answering this question is illustrated by recent studies documenting a more pronounced regression of preestablished tumors following simultaneous down-regulation of cathepsin B and MMP9 *(179)* as well as MMP9 and uPAR *(180, 181)* using direct intra-tumoral injections of interfering RNAs (RNAi), when compared with targeting either protease alone. Nonetheless, although these approaches are promising, future studies should take into consideration the possibility that complete loss of one enzymatic activity may

replace by "pre-established"

be compensated for by activation of alternative pathways. For instance, following genetic elimination of cathepsin B, tumor cells have been found to induce cathepsin X expression, resulting in a partial compensation for loss of cathepsin B-mediated effects *(168)*. Taken together, the net proteolytic activity of neoplastic tissues can no longer be understood without taking into consideration the cascade of protease activities and the potential interconnections that form – that is the flow of proteolytic activities within the protease network as a whole.

4. Proteases as Therapeutic Agents

Recent studies exploited the unique localization of specific protease pathways, such as proteases that are tethered to cell surfaces, including uPA, thus directing proteolysis to discrete focal areas, versus intracellular proteolytic activities within lysosomes, such as cathepsins. These approaches have recently been used to direct activation of nontoxic "prodrugs" selectively to tumor sites *(182)*. More specifically, incorporation of a tripeptide specifier that is recognized exclusively by tumor-associated plasmin initiates release of chemotherapeutic agents, such as doxorubicin (Dox), from its prodrug form selectively in the vicinity of tumor cells, as opposed to systemic administration of Dox, which limits its activation due to the presence of physiological plasmin inhibitors, including α_2-antiplasmin and α_2-macroglobulin *(182)*. Using such novel approaches, Dox has been shown to exert its cytotoxic and/or cytostatic effects locally while restricting cardiotoxicity, a side effect that may clinically limit Dox dosage intake. It follows that similar interesting strategies have been applied to design prodrugs that are activated in tumor cells over-expressing selective protease pathways, including cathepsin B. For instance, Panchal et al. *(183)* triggered selective apoptosis of tumor cells by engineering prodrugs that are pore forming toxins, known as "prolysins." Once activated selectively by malignant tumor cells expressing high levels of membrane-associated cathepsin B, α-hemolysin is released, inducing selective permeabilization of tumor cells and ultimately, cell death.

Furthermore, recent novel advances in anti-cancer therapies took advantage of selective expression of the anti-apoptotic protein survivin in malignant ovarian cells to specifically activate expression of cytotoxic T lymphocyte-derived proapoptotic proteases, such as granzyme B *(184)*. Driven by the *survivin* promoter, active granzyme B not only reduced tumor incidence and size of xenografted human ovarian carcinoma in nude mice, but also prevented metastatic

spread *(184)*. These recent studies illustrate the utility of proteases that are normally employed by immune cells to eliminate tumor cells in designing future therapeutics. Taken together, the complexity by which proteases may interact must be taken into account to delineate the physiological as well as pathological roles of proteases, as opposed to merely investigating individual proteases.

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Chapter 9
Proteolytic Pathways: Intersecting Cascades in Cancer Development

Nesrine I. Affara and Lisa M. Coussens

Abstract Matrix remodeling proteases, including metalloproteinases, serine proteases, and cysteine cathepsins, have emerged as important regulators of cancer development due to the realization that many provide a significant protumor advantage to developing neoplasms through their ability to modulate extracellular matrix metabolism, bioavailability of growth and proangiogenic factors, regulation of bioactive chemokines and cytokines, and processing of cell–cell and call–matrix adhesion molecules. While some proteases directly regulate these events, others contribute to cancer development by regulating posttranslational activation of other significant protease activities. Thus, understanding the cascade of enzymatic activities contributing to overall proteolysis during carcinogenesis may identify rate-limiting steps, or pathways, that can be targeted with anticancer therapeutics. This chapter reviews recent insights into the complexity of roles played by extracellular and intracellular proteases that regulate tissue remodeling accompanying cancer development and focuses on the intersecting proteolytic activities that amplify protumor programming of tissues to favor cancer development.

Introduction

It is well established that cancer arises as a consequence of genetic alterations in genes that provide a survival and/or proliferative advantage to mitotically active cells. By studying mouse models of de novo cancer development, it is now clear that genetically altered neoplastic cells co-opt important physiologic host–response processes, that is, extracellular matrix (ECM) remodeling, angiogenesis, activation/recruitment of innate, and adaptive leukocytes (inflammation), early during cancer

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development to favor their own survival. While it was initially believed that matrix remodeling proteases merely regulated migration and/or invasion of neoplastic cells into ectopic tissues, it is now clear that their more significant contribution has to do with regulating the bioactivity of a diverse array of growth factors, chemokines, soluble and insoluble matrix molecules that regulate activation and/or maintenance of overall tissue homeostasis, as well as inflammatory and angiogenic programs in pathologically damaged tissues, including cancer (Van Kempen et al. 2006). Moreover, it has also become clear that in vivo, many critical proteolytic cofactors for cancer development derive from activated stromal cells and thus reinforces the concept that cancer development requires reciprocal interaction between genetically altered neoplastic cells with activated diploid stromal cells and the dynamic microenvironment in which they both live (Bissell and Aggeler 1987, Bissell and Radisky 2001). Recent advances in activity-based profiling of protease function (Blum et al. 2005, 2007; Salomon et al. 2003; Sieber and Cravatt 2006; Sloane et al. 2006) have enabled tracking the distribution and magnitude of proteolytic activities in cells and tissues (Kato et al. 2005). Together with insights gained from examining individual protease gene functions in mouse models of de novo carcinogenesis, have emerged insights into the multitude of enzymatic activities that participate in tissue remodeling associated with cancer development (Egeblad and Werb 2002, Lopez-Otin and Overall 2002). Recently, sequencing of the human genome enabled characterization of the human degradome, which has been found to consist of at least 569 proteases and homologues that belong to various classes, including metalloproteinases, serine proteases, and cysteine cathepsins (Lopez-Otin and Matrisian 2007; Fig. 9.1 and *see* also Chap. 1 by Puente, Ordonez and López-Otín, this volume). For many of these enzymes, their most significant protumor activity may lie in their ability to posttranslationally regulate other proteases initially secreted as either inactive zymogens or sequestered by matrix in nonactive forms (Lopez-Otin and Matrisian 2007). This realization has led to the notion that embedded within tissues are complex, interconnecting protease networks that, depending on the tissue perturbation, selectively engage specific protease amplification circuits (Lopez-Otin and Overall 2002). These coordinated efforts regulate overall tissue homeostasis, response to acute damage, and subsequent tissue repair, as well as contribute to the pathogenesis of chronic disease states such as cancer.

Proteases Implicated in Cancer Development

Requisite for neoplastic, vascular, or inflammatory cell invasion during tumorigenic processes are the remodeling events that occur within the stroma or the ECM and on cell surfaces. ECM-remodeling proteases are universally expressed during tumor progression and metastasis, where in addition to interacting with ECM and cell surface substrates, many in turn regulate activation of other proteases initially secreted as inactive zymogens or sequestered in ECM (Dano et al. 1999, DeClerck et al. 2004, Egeblad and Werb 2002). Several classes of ECM-remodeling proteases have been

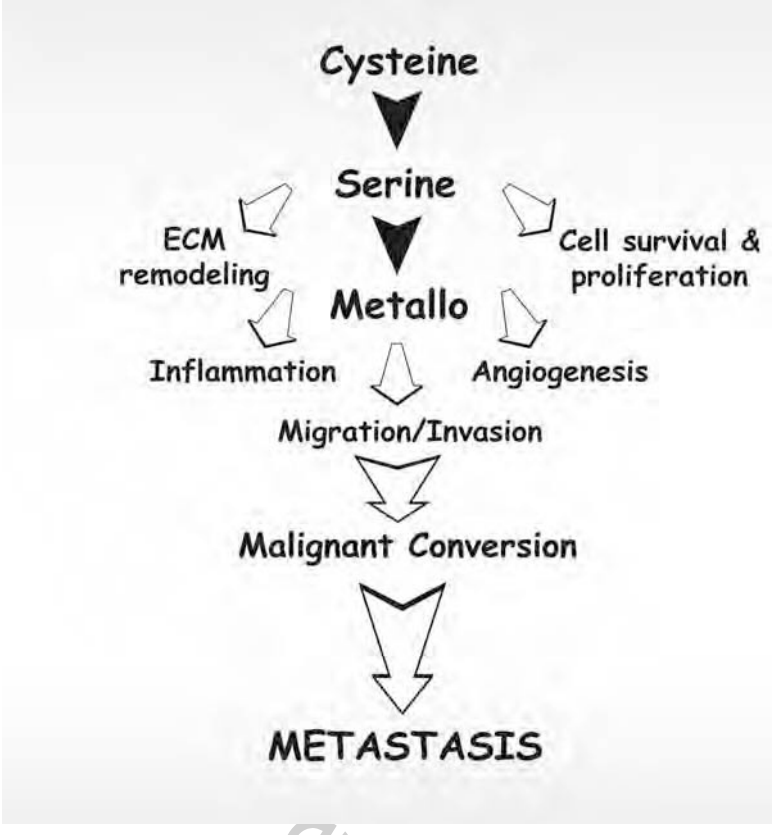

Fig. 9.1 Proteases act as critical cofactors for cancer development. Proteases belonging to several catalytic classes, including metalloproteinases, serine, and cysteine proteases, have emerged as important regulators of tissue remodeling, inflammation, angiogenesis, and acquisition of invasive capabilities, processes that accompany and potentiate cancer development

identified (metallo-, serine, and cysteine), some have emerged as important regulators of tissue remodeling, inflammation, and angiogenesis accompanying cancer development. In epithelial tumors, a majority of ECM-remodeling proteases are made by activated stromal cells, a large percentage of which being infiltrating leukocytes such as mast cells, immature myeloid cells, monocytes, macrophages, granulocytes, and lymphocytes (Van Kempen et al. 2006). In vivo assessment of individual protease gene functions have indeed identified some proteases as significant cofactors for cancer development due to their ability to regulate important aspects of neoplastic progression, others are significant in that they set in motion interconnecting protease cascades resulting in amplification of enzymatic activity of "terminal" proteases, such as matrix metalloproteinase (MMP)-9 (Fig. 9.2). While these cascades of activation important for carcinogenesis resemble those regulating coagulation (Hoffman and Monroe 2005) and/or complement (Carroll

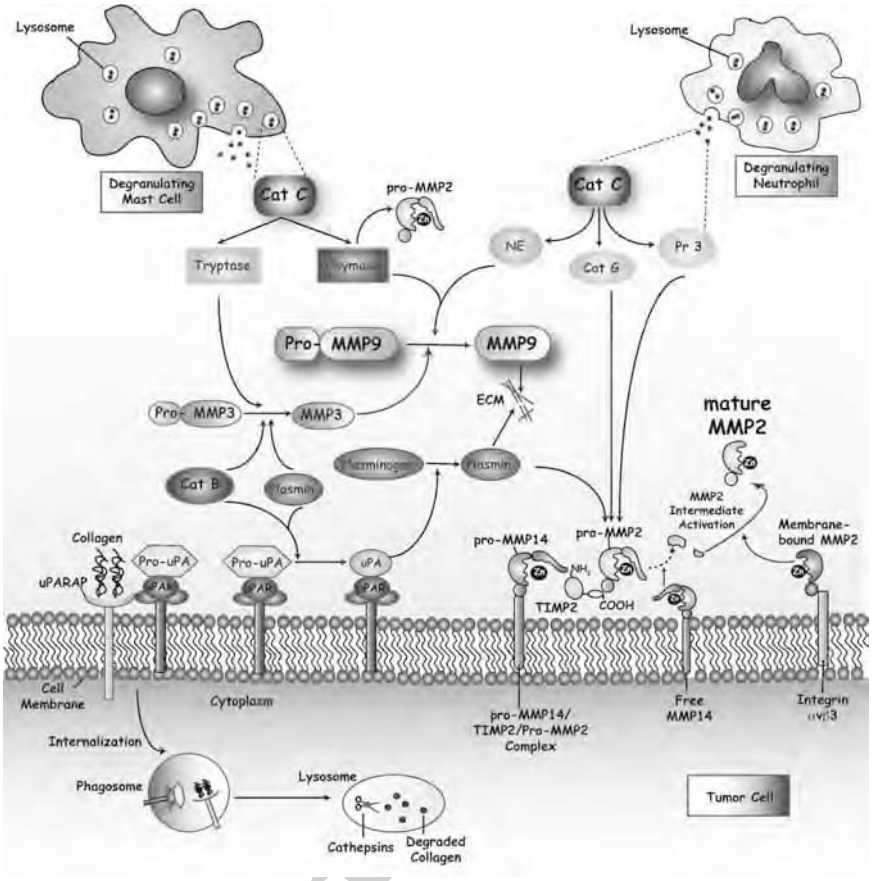

Fig. 9.2 Intersecting protease pathways during neoplastic progression. Perturbation of extracellular matrix (*ECM*) components through proteolysis during tumor progression results from the activity of combined protease pathways belonging to diverse proteolytic enzyme systems, including matrix metalloproteinases (*MMPs*), serine proteases, such as uPA, plasmin, mast cell chymase, mast cell tryptase, neutrophil elastase, and proteinase 3, and cathepsins, such as cathepsin C. Rather than functioning individually, each protease functions as a "signaling molecule" that exerts its effects as part of a proteolytic pathway, where proteases potentially interact and activate other proteases in a cascade-like manner, culminating in amplification of enzymatic activity of "terminal" proteases, such as MMP-9

2004), in vivo experimental studies in mouse models have revealed organ- and tumor type-specific regulation of protease bioactivities, as well as involvement of proteases emanating from multiple enzymatic classes, that is, cysteine, serine, and metallo. In the sections that follow, we discuss a significant role for MMP-9 during tumor progression in multiple organ sites, and examine the diversity of proteases whose bioactivity result in amplification of MMP-9-mediated effects in tumor tissue.

Metalloprotease Activation and Function During Cancer Development

MMPs, also known as matrixins, are a family of zinc-dependent endopeptidases that facilitate neoplastic progression not only by degrading structural components of the ECM but also by triggering release of growth and angiogenic factors sequestered by neoplastic tissues and by processing of cell–cell and cell–matrix adhesion molecules (Egeblad and Werb 2002). To date, 23 vertebrate MMPs have been identified and classified into distinct categories based on domain structure and substrate specificity (Egeblad and Werb 2002, Puente et al. 1996). Bioactivity of MMP function is controlled posttranslationally. Secreted MMPs (with the exception of stromelysin-3/MMP-11) remain as inactive zymogens, requiring enzymatic and/or autolytic removal of propeptide domains. Once activated, however, MMPs are further regulated by two major types of endogenous inhibitors: α_2-macroglobulin and tissue inhibitors of metalloproteinases [TIMPs; reviewed in Egeblad and Werb (2002)]. Bioactivity of TIMPs is further regulated posttranslationally where some are known to be inactivated by serine protease cleavage (Frank et al. 2001).

Serine proteinases, such as plasmin or urokinase-type plasminogen activator (uPA), neutrophil elastase, mast cell chymase, and trypsin, cleave propeptide domains of secreted pro-MMPs and consequently induce autocatalytic activation of MMP-1, -3, and -9 (Egeblad and Werb 2002). Some activated MMPs can further activate other pro-MMPs. For example, MMP-3 activates pro-MMP-1 and pro-MMP-9, whereas pro-MMP-2 is resistant (Egeblad and Werb 2002). Thus, some serine proteinases act as initiators of activation cascades regulating bioactivity of pro-MMPs in vivo.

Cell-mediated activation mechanisms have also been identified, most notably represented by a plasma membrane-associated ternary complex formed by pro-MMP-2, TIMP-2, and the transmembrane-spanning MMP [MMP-14/MT1-MMP; Itoh et al. (2001), Wang et al. (2000), Worley et al. (2003)] that is activated intracellularly (Pei and Weiss 1995, Yana and Weiss 2000). Several advantages to having degradative enzymes in a bound state at the cell surface have been proposed. Namely, bound proenzymes may be more readily activated and the bound enzymes generated may be more active than the same enzymes found in the soluble phase. Bound enzymes may be protected from inactivation by inhibitors, binding of an enzyme to a cell surface may provide a means of concentrating components of a multistep pathway, thereby increasing rate of reactions. Immobilizing enzymes on the cell surface or in matrix may provide a means of restricting activity of an enzyme so that substrates only in the vicinity of the cell or adjacent matrix components are degraded. Hence, activation at the cell surface links MMP expression with proteolysis and invasion and may actually provide the most significant control point in MMP activity. Au1

To address the functional roles for MMPs during cancer development, tumor-prone organ-specific transgenic mouse models harboring homozygous null gene

deletions in individual MMPs have been utilized. Using a transgenic mouse model of multistage skin carcinogenesis where the early region genes of human papillomavirus type 16 (HPV16) are expressed as transgenes under control of the human keratin 14 (K14) promoter, for example, K14-HPV16 mice (Coussens et al. 1996), genetic elimination of MMP-9 significantly reduced the incidence of carcinomas in K14-HPV16 mice, while in contrast, reconstitution of K14-HPV16/MMP-9^{null} mice with wild type bone marrow-derived cells restored characteristics of neoplastic development and tumor incidence to levels similar to control HPV16 mice (Coussens et al. 2000). More specifically, the cellular source of cells supplying MMP-9 in neoplastic tissues was predominately chronically activated innate immune cells, whose infiltration coincided with development of angiogenic vasculature in premalignant skin (Coussens et al. 2000). Similarly, angiogenesis and tumor development were significantly inhibited during pancreatic islet carcinogenesis (Bergers et al. 2000) and cervical carcinogenesis (Giraudo et al. 2004) when MMP-9 was either genetically deleted or inhibited pharmacologically. Similarly, in each of these distinct tissue microenvironments, infiltrating leukocytes were the predominant sources of MMP-9 (Bergers et al. 2000, Coussens et al. 2000, Giraudo et al. 2004). During development of ovarian carcinomas using a xenograph model (Huang et al. 2002), as well as during skin carcinogenesis (Coussens et al. 2000) and neuroblastoma development (Jodele et al. 2005), reconstitution of MMP-9-deficient mice with MMP-9-proficient bone marrow-derived cells restored cellular programs necessary for development of angiogenic vasculature, tissue remodeling, and overt tumor development, thus implicating leukocyte-derived MMP-9 as a significant cofactor for cancer development.

Proangiogenic roles for leukocyte-derived MMP-9 are now well accepted. During islet carcinogenesis, although vascular endothelial growth factor (VEGF) is constitutively expressed in normal β-cells and at all stages of islet carcinogenesis, it only becomes bioavailable for interaction with its receptor on microvascular endothelial cells following infiltration of leukocytes expressing MMP-9, thereby triggering activation of angiogenic programs (Bergers et al. 2000). During development of experimental neuroblastomas, MMP-9 may regulate bioavailability of VEGF, but also regulates pericyte recruitment to developing angiogenic vessels, thus inducing stabilization of newly formed tumor vasculature (Chantrain et al. 2004, 2006). An additional line of evidence supporting a role for MMP-9 in promoting neovascularization comes from studies reporting the unique ability of MMP-9 to induce release of soluble kit-ligand, that thereby initiates mobilization of hematopoietic stem cells/progenitor cells in bone marrow (Heissig et al. 2002, Jodele et al. 2005). Au2

Moreover, while MMP-9 induces a tissue microenvironment that is permissive not only for primary tumor development but its bioactivities also regulate secondary metastasis formation. Some clues into the later aspects of events regulating metastasis have implicated MMP-9 made by macrophages and alveolar VEGF receptor (VEGFR1)$^{+}$ endothelial cells in microenvironmental remodeling necessary for metastatic cell survival in lung (Hiratsuka et al. 2002). Using a mouse model of experimental metastasis formation, Hiratsuka et al. (2002) reported that

following recruitment to sites of primary tumor growth, macrophages circulate to distal organs. Distal organs exhibiting low-level expression of VEGFR1 fail to induce MMP-9 in response to leukocyte presence and are therefore not suitable environments for subsequent metastatic cell growth. In contrast, distal organs that are VEGFR1-positive and contain a population of endothelial cells capable of inducing expression of MMP-9 above that supplied by circulating macrophages are "fertile" sites for productive metastatic growth. While induced expression of the VEGFR1 ligand VEGF-A does not appear to be involved, presence of an active VEGFR1 tyrosine kinase domain is necessary; thus, it seems reasonable that activated MMP-9 releases matrix-sequestered VEGF-A rendering it bioavailable for interaction with its receptors as has been reported by Bergers and colleagues (Bergers et al. 2000), thus, stimulating efficient vascular remodeling and angiogenesis necessary for metastatic cell growth and survival. Studies by Matrisian and colleagues have demonstrated MMP-9 derived from inflammatory cells (possibly neutrophils) present in premetastatic lung facilitates survival/establishment of early metastatic cells, but not growth of metastatic foci (Acuff et al. 2006), while MMP-9 derived from Kupffer cells in liver parenchyma, and not from bone marrow-derived cells, facilitate ability of metastatic colon cancer tumor foci to grow (Gorden et al. 2007). Taken together, these findings indicate that mechanisms by which premetastatic niches enhance metastatic outgrowth are organ and cancer-type specific.

In addition to MMP-9, other MMPs have also emerged as important cofactors in cancer development. For instance, studies using MMP gene knockout mice have indicated a key role of MMP-7 in the development of intestinal adenomas in the multiple intestinal neoplasia (Min) mouse model of intestinal neoplasia (Wilson et al. 1997). Genetic elimination of MMP-11 (stromelysin 3) resulted in a decreased tumor incidence and tumor size in 7,12-dimethylbenzanthracene (DMBA)-induced carcinomas (Masson et al. 1998). In contrast to the role of MMPs in promoting tumor progression, studies using MMP-3 (stromelysin 1)-deficient mice revealed a protective role for MMP-3 during chemically induced squamous cell carcinoma development (McCawley et al. 2004). Similarly, loss of MMP-8 (collagenase 2) enhanced rather than reduced skin tumor susceptibility in MMP-8-deficient male mice (Balbin et al. 2003). In contrast, while MMP-14-deficient mice have not been specifically examined using de novo models of cancer development, the role of MMP-14 as a cancer cofactor is undisputed. MMP-14, alone or in concert with MMP-2, has been found to activate procollagenase-3 (pro-MMP-13) (Knauper et al. 1996), that in turn mediates degradation of ECM components, including proteoglycans as well as type II collagen (Fosang et al. 1996). MMP-14 and MMP-2 have also been shown to release cryptic fragments of laminin-5 $\gamma 2$ chain domain III, which due to presence of epidermal growth factor (EGF)-like repeats, binds to EGF-receptor on tumor cells, thus activating downstream signaling events that lead to tumor cell motility (Giannelli et al. 1997, Koshikawa et al. 2000). Taken together, the coordinated expression of MMP-14, MMP-2, and MMP-13 induces formation of a cascade of zymogen activation in close proximity to the tumor cell surface, resulting in amplification of proteolysis within pericellular tumor microenvironments.

It is not surprising that MMPs have attracted significant attention as anticancer therapeutic targets. Unfortunately, clinical evaluation of MMP inhibitors revealed no efficacy in patients suffering from the advanced stages of various types of cancer (Coussens et al. 2002). These failed clinical experiments have nonetheless enabled revisiting of the upstream regulatory mechanisms controlling activation of important proteases, like MMP-9, that play a clear and undisputed role in cancer development (Fig. 9.2). Active MMP-9 represents a terminal protease target whose proteolytic activity is amplified by several proteolytic pathways that converge or act in parallel to activate the latent proform of the enzyme, and thus indicating that anti-protease-based therapeutics may achieve better efficacy when targeting a "pathway" as opposed to a single class or single species of enzyme(s).

Serine Protease Regulation of MMP Activity During Cancer Development

Several serine proteases have been implicated as important regulators of cancer development, some of which are known regulators of MMP-9 bioactivity. Some of these include enzymes involved in regulating activation of plasminogen [urokinase-type and tissue-type plasminogen activators, uPA and tPA, respectively; *see* Chap. 10 by Bugge and Chap. 11 by Almholt et al., this volume; Bugge et al. (1998)], as well as serine proteases stored in secretory lysosomes of leukocytes, namely mast cell chymase (Coussens et al. 1999), mast cell tryptase (Coussens et al. 1999), and neutrophil elastase [NE; Starcher et al. (1996)]. Au3

Serine proteases are synthesized as inactive zymogens whose activation involves a two-step mechanism (Caughey 2002, Reiling et al. 2003). Following synthesis and passage through the endoplasmic reticulum where signal peptides are removed, the proprotease containing an "activation dipeptide" is generated characterized by presence of two amino-terminal residues that blocks substrate access to the catalytic site cleft, thereby maintaining the latent state and preventing premature protease activation. In addition, serine protease proteolytic activity is tightly regulated by low pH of the environment typical of secretory granules.

Plasminogen Activators

Several mechanistic studies have reported that the uPA/plasmin proteolytic cascade functionally contributes to neoplastic progression, including acquisition of a migratory and invasive phenotype by tumor cells, as well as remodeling of ECM components via activation of a number of MMPs, such as MMP-9 (Ramos-DeSimone et al. 1999). Enzymatic activity of plasmin is tightly regulated by two plasminogen activators, uPA and tPA (tissue-type plasminogen activator). Initiation of plasmin activation occurs following binding of uPA to its receptor uPAR (urokinase receptor), a gycosyl-phosphathidylinosityl (GPI)-anchored cell mem-

brane protein that has been found to localize at discrete focal contacts (Pollanen et al. 1988). Subsequently, binding of uPA to uPAR catalyzes conversion of plasminogen to its active form, plasmin. In contrast, uPAR-bound pro-uPA is also activated by plasmin, which in turn results in a feedback pathway that accelerates plaminogen activation (Ellis et al. 1991; Fig. 9.1). Once active, cell-bound plasmin contributes to ECM remodeling by directly activating pro-MMPs, including pro-MMP-1, -2, -3, and -9 (Hahn-Dantona et al. 1999, He et al. 1989, Monea et al. 2002, Ramos-DeSimone et al. 1999).

Maintenance of this cascade depends upon the balance between uPAR-bound uPA proteolytic activity and endogenous inhibitors, including plasminogen activator inhibitor-1 (PAI-1) (Wun and Reich 1987). PAI-1 not only regulates proteolytic activity of uPA but also induces rapid internalization of the uPA/PAI-1/uPAR complex (Conese and Blasi 1995), thus regulating levels of cell surface-bound uPA as well. Taken together, through focally localized uPA/uPAR proteolytic activity at the cell surface, plasmin is considered a key event in conferring tumor cells with their ability to migrate through fibrinous matrices.

Mast Cell Serine Proteases

Mast cells represent a rich source of serine protease activity (uPA, chymases, and tryptases) stored in secretory granules that is released into the extracellular milieu following activation/degranulation in response to cross-linking of FcεRI, a high affinity receptor for immunoglobulin E (IgE) (Blank and Rivera 2004) as well as by mechanisms including components of the complement system (C3a and C5a) (el-Lati et al. 1994), neuropeptides such as substance P (Karimi et al. 2000), cytokines including stem cell factor (Hogaboam et al. 1998), as well as engagement of the Toll-like receptors (Kulka et al. 2004), that induce mast cell activation independently of IgE. While tryptases are known to cleave substrates at the carboxyl-terminal side of basic amino acids, chymases in contrast exert chymotrypsin-like activity and cleave peptides at the carboxyl-terminal side of aromatic amino acids (Schechter and Berger 1967, Schechter et al. 1986). To date, one human chymase gene belonging to the α-chymase family has been identified, while rodents express α-chymase (murine mast cell protease-5/mMCP-5), as well as several β-chymases [mMCP-1, -2, and -4; (Tchougounova et al. (2003)]. Interestingly, genetic elimination of mMCP4 completely abolishes chymotrypsin-like activities in peritoneal cells and cutaneous tissue (skin) isolated from mMCP-4-deficient mice (Tchougounova et al. 2005), demonstrating that mMCP-4 is the major source of stored chymotrypsin-like activity in these tissues. In contrast, mast cell tryptases in rodents include mast cell protease-6 (mMCP-6) and mMCP-7. While mMCP-7 is released into the circulation following mast cell degranulation, mMCP-6 is released into the vicinity of degranulated mast cells (Ghildyal et al. 1996). Recently, a novel mast cell tryptase, denoted mMCP-11/mastin, was also reported in dogs, pigs, and mice (Wong et al. 2004). In humans, tryptases fall into two major categories,

including membrane-anchored tryptases, such as γ-tryptase, and soluble tryptases, such as α-, β-, and δ-tryptases, the latter that has also been termed mMCP-7-like tryptase, given the similarity with mMCP-7 genetic organization. In particular, only β-tryptase plays important roles following mast cell secretion since α- and β-tryptases are secreted in their inactive zymogen forms due to the presence of catalytic domain defects in addition to the presence of propeptide mutations [reviewed in Caughey (2007)].

The functional significance of mast cell-derived chymases and tryptases in neoplastic progression has been recently appreciated. Mast cell chymase has been shown to elicit proinflammatory effects through its ability to mediate recruitment of granulocytes into inflamed tissues (He and Walls 1998). Indeed, injection of human chymase into skin of guinea pigs resulted in a significant increase in neutrophil influx as well as increased vascular permeability (He and Walls 1998). Although many enzymes have been shown to activate pro-MMP-9, mast cell-derived mMCP-4 plays a critical role in activation of MMP-9, given that only the proform of MMP-9 was detected in tissue extracts isolated from mMCP-4-deficient mice (Tchougounova et al. 2005). Moreover, levels of active MMP-2 were significantly lower in the absence of mMCP-4 as compared to tissues from wild-type mice, indicating that mMCP-4 is important but not essential for activation of pro-MMP-2 in vivo (Tchougounova et al. 2005). In contrast, α-chymase has also been reported to cleave and inactivate free TIMP-1, in addition to TIMP-1 when bound in a complex with pro-MMP-9, thus enabling conversion of inhibited MMP-9 to active MMP-9 (Frank et al. 2001). Thus, chymase possesses indirect proangiogenic activities via regulating release of sequestered VEGF from the matrix following activation of MMP-9 (Coussens et al. 1999). In addition, by evaluating skin, heart,, and lung tissues in mMCP-4 homozygous null mice, a major role of mast cell-derived chymase in regulating turnover of connective tissue components, including thrombin, fibronectin, and collagen, has been revealed (Tchougounova et al. 2005).

In contrast to chymases, mast cell tryptases have been implicated in cancer development due to their ability to act as direct mitogens for stromal fibroblasts (Hartmann et al. 1992, Ruoss et al. 1991) and epithelial cells (Cairns and Walls 1996), as well as their ability to activate MMP-9 indirectly through their ability to initially activate pro-MMP-3 (Gruber et al. 1989; Fig. 9.1). Furthermore, mast cell-derived tryptases have been found to modulate neoplastic microenvironments during skin carcinogenesis by stimulating synthesis of α_1 procollagen mRNA and proliferation of dermal fibroblasts (Coussens et al. 1999). Tryptases also act as potent proinflammatory factors, given that injection of mMCP-6, but not mMCP-7, induced neutrophil infiltration into peritoneal cavities (Huang et al. 1998). Although the mechanism by which mMCP-6 mediates accumulation of neutrophils in inflamed tissues remains to be determined, recent studies indicate that tryptases induce leukocyte recruitment indirectly by stimulating chemokine release, such as IL-8, from endothelial and epithelial cells (Cairns and Walls 1996, Compton et al. 1999). Taken together, these findings indicate that mast cell-derived tryptases may functionally contribute to ECM remodeling and inflammation that accompanies cancer development.

While specific gene knock-outs of murine chymases and/or tryptases have yet to assessed in a de novo tumor model, their role in cancer appears clear; thus, mechanisms regulating their activity in neoplastic tissues has been examined. In contrast to most MMPs that are secreted as zymogens requiring pericellular activation, mast cell proteases are stored in mast cell secretory granules in their mature, enzymatically active forms ready for exocytic release. Upstream intracellular activators of mast cell serine proteases include cathepsin C (Wolters et al. 2001), a cysteine protease particularly abundant in mast cell secretory granules, thus implicating presence of a cascade of proteolytic activations (Fig. 9.2), that may serve as potential therapeutic intervention strategies during tumor progression.

Neutrophil-Derived Serine Proteases

Neutrophil elastase (NE) is a serine protease transcriptionally activated during early myeloid development and subsequently stored in azurophilic granules of neutrophils (Fouret et al. 1989, Zimmer et al. 1992). While the role of NE in pulmonary disorders, including emphysema and fibrosis, has been well documented (Chua and Laurent 2006), much less is known about its role in cancer. Interest in NE during neoplastic processes stems from its ability to directly modulate ECM components or indirectly through initiation of protease cascades that culminate in activation of MMPs, including pro-MMP-9 (Ferry et al. 1997). In addition, NE has been found to regulate inflammatory processes by enhancing neutrophil migration into inflamed tissues (Nakamura et al. 1992). Through its ability to localize to plasma membranes following exocytosis, membrane-bound NE facilitates transendothelial migration of neutrophils (Cepinskas et al. 1999).

Early studies implicated NE as a key effector molecule regulating neutrophils, given its potent ability to mediate intracellular clearance of bacteria (Belaaouaj et al. 1998). More recently, a novel antimicrobial mechanism including secretion of extracellular structures, also termed neutrophil extracellular traps (NET), has been reported (Fuchs et al. 2007). NETs contain chromatin and sequestered neutrophil-derived granule proteases that enable neutrophils to deliver high concentration of proteolytic enzymes, including NE, to mediate bacterial clearance extracellularly (Brinkmann et al. 2004, Fuchs et al. 2007).

Recent clinical reports have correlated elevated NE expression with poor survival rates in patients with primary breast cancer (Akizuki et al. 2007) and nonsmall cell lung cancer (Yamashita et al. 1996), as well as having recently been found to initiate development of acute promyelocytic leukemia by mediating direct catalytic cleavage of PML-RARα, a protein generated by a chromosomal translocation fusing promyelocytic leukemia (PML) and retinoic acid receptor-α (RARα) genes (Lane and Ley 2003).

Experimentally, localization of active NE to the outer surface of the plasma membrane enables neutrophil transmigration in vivo (Young et al. 2007). Using in vivo intravital microscopy, recent studies revealed that neutrophil adhesion to

postcapillary venules and emigration out of vasculature were attenuated in the presence of selective NE inhibitors (Woodman et al. 1993). This role is further supported by studies using a mouse model of acute experimental arthritis that found reduced neutrophil infiltration into subsynovial tissue spaces in NE-deficient mice (Sato et al. 2006), which were also found to exhibit reduced incidence of ultraviolet B- and chemically (Benzopyrene)-induced skin tumors (Starcher et al. 1996). Whether reduced tumor incidence was due to impaired NE-mediated cleavage of ECM substrates, deficient MMP-9 activation, or diminished cleavage of NE substrates such as ECM components or E-selectin on endothelial cells (Nozawa et al. 2000) remains to be established. In addition, genetic elimination of both NE and cathepsin G (a cysteine protease) in mice completely attenuated neutrophil emigration to sites of inflammation in response to zymosan particles in an air-pouch model of inflammation (Sato et al. 2006). However, absence of NE did not alter neutrophil recruitment to inflamed tissues in response to lipopolysaccharide (Hirche et al. 2004). While neutrophils migrated normally to sites of bacterial infection in NE-deficient mice, their ability to initiate intracellular killing of gram-negative bacteria was altered (Young et al. 2004), thus indicating some degree of specificity for recruitment and response regulated by NE.

The ability of NE to differentially regulate neutrophil recruitment and presence in "damaged" tissues, while directly significant for acute inflammatory responses, is also significant in the context of the role of neutrophils in resulting remodeling of matrix in tissues. Neutrophil-derived proteases have been identified as important regulators of insoluble elastin (Baugh and Travis 1976), a structural component of tissues such as blood vessels, skin, and lung, in addition to hydrolysis of other ECM components, including fibronectin (McDonald and Kelley 1980), proteoglycans, and type IV collagen catabolism (Mainardi, Dixit and Kang et al. 1980, Sato et al. 2006, Cepinskas et al. 1999). Some of these ECM molecules are direct substrates for NE, while others are substrates for enzymes whose bioactivity is regulated by NE.

Central to the proposed roles of NE in tumorigenesis is the mechanism of NE activation and identification of potential target substrates. NE is synthesized as an inactive zymogen requiring posttranslational removal of the amino-terminal dipeptide for enzymatic activation (Adkison et al. 2002). Propeptide cleavage occurs prior or during transport of NE to neutrophil azurophil granules through the catalytic activity of cathepsin C (Fig. 9.2). Following activation of neutrophils by various cytokines and chemoattractants, NE is secreted in its catalytically active form. One mechanism by which NE resists inhibition by circulating proteinase inhibitors, including α_1-antitrypsin (Owen et al. 1995), is by localizing to neutrophil cell surface following fusion of primary granules with plasma membranes during exocytosis (Lee and Downey 2001).

NE can indirectly modulate structural components of tumor ECM and facilitate tumor cell migration by activating MMPs. Following binding of pro-MMP-2 to membrane-anchored MT1-MMP/MMP-14, pro-MMP-2 becomes susceptible to activation by NE, as opposed to soluble pro-MMP-2 that is resistant to activation by NE (Shamamian et al. 2001). Furthermore, NE has also been shown to activate pro-MMP-3 (Okada and Nakanishi 1989), as well as pro-MMP-9 (Ferry et al.

1997). In turn, MMP-9 can cleave the NE inhibitor α1-antitrypsin, and thereby indirectly enhance NE enzymatic activity (Liu et al. 2000).

Taken together, these observations extend the role of NE to a modulator of inflammatory responses, given its ability to facilitate neutrophil extravasation into inflamed tissues by directly acting on ECM components such as elastin (Baugh and Travis 1976). Furthermore, NE indirectly induces ECM remodeling by activating MMPs (Ferry et al. 1997), and thus promoting tumor cell migration and invasion. Following excessive neutrophil influx at sites of inflammation, catalytically active NE is rapidly released from azurophil granules, along with terminal proteases including pro-MMP-9 stored in its zymogen form in neutrophil gelatinase granules. In turn, catalytically active NE within tumor microenvironments induces activation of secreted pro-MMP-9 (Fig. 9.2). One way NE may induce amplification of MMP-9-mediated effects is by catalyzing activation of pro-MMP-9 originating from other cell populations, including infiltrated mast cells. Thus, neutrophil-derived NE can significantly contribute to net tumor proteolysis. Moreover, current insights regarding protease degradomics reveal that in addition to targeting distinct matrix substrates, serine proteases exhibit partially overlapping target substrate profiles, such as activation of pro-MMP-9, whose net activity is significantly amplified during pathological processes and following simultaneous activation of various serine protease circuits originating from different cellular compartments, such as mast cells and neutrophils (Fig. 9.1). Alternatively, despite the notion that these protease pathways may be individually redundant, the above observations support the notion that serine proteases profoundly influence neoplastic progression by acting collectively.

Cysteine Cathepsin Proteases in Cancer Development

Cathepsins are lysosomal cysteine proteases that belong to the papain-like superfamily, characterized by presence of an active site cleft in which amino acid residues cysteine and histidine constitute the catalytic ion pair (Turk et al. 2001). Human cathepsins comprise 11 members: cathepsins B, C, H, F, K, L, O, S, V, W, and X/Z. While several family members have been identified as important regulators of cancer development, we focus here on cathepsin C due to the significant role it plays in regulating multiple serine proteases that together regulate important immune-based aspects of cancer development include regulating overall MMP-9 bioactivity.

Cathepsin proteolytic activity is regulated at various levels. All members of the cathepsin family are synthesized as zymogens, sharing a signal peptide and propeptide sequence removed at maturation (Vasiljeva et al. 2007). Interestingly, a residual portion of the propeptide, termed the exclusion domain, remains bound to the catalytic part of active cathepsin C (Dolenc et al. 1995) that contributes to formation and stabilization of the tetrameric structure of the mature enzyme (Cigic et al. 2000). In vitro studies indicate that procathepsin C cannot autolytically activate (Dahl et al. 2001), but identity of cathepsin C activators are yet to be determined.

While most cathepsins act as endopeptidases, cathepsin C, also known as dipeptidyl peptidase I (DPPI), represents the only exception by acting as a dipeptidyl aminopeptidase, cleaving two-residue units from the N-terminus of a polypeptide chain (McGuire et al. 1992). Localization of active sites of cathepsin C to the external surface of the protein confers cathepsin C with an advantage of hydrolyzing diverse groups of chymotrypsin-like proteases in their native state, regardless of size (Turk et al. 2001), as opposed to other oligomeric proteases, including tryptases, where active site are located inside of the protein (Hallgren and Pejler 2006).

While cathepsins were initially thought to only mediate terminal intracellular protein degradation within lysosomes, it is now evident that individual cathepsins exert diverse biological functions, including interstitial thrombin and fibronectin metabolism, cytotoxic lymphocyte-mediated apoptotic clearance of virus-infected and tumor cells (Shresta et al. 1998), survival from sepsis (Mallen-St Clair et al. 2004) and experimental arthritis (Adkison et al. 2002). Using cathepsin C homozygous null mice, cathepsin C has been found to activate several serine proteases, thus supporting its role as an important upstream regulator of multiple proteolytic events. In the absence of cathepsin C, cytotoxic T lymphocyte-derived granzymes A and B were found to be inactive and present in their proforms (Pham and Ley 1999). Similarly, cathepsin C has been found to be essential for intracellular activation of neutrophil-derived NE, cathepsin G and proteinase 3 (Adkison et al. 2002). In contrast, although cathepsin C has been shown to activate both human mast cell pro-α-chymase and pro-β-tryptase in vitro (Muramatsu et al. 2000, Sakai et al. 1996), cathepsin C was essential for activation of only mouse mast cell chymase in vivo, as opposed to mMCP-6, a mouse tryptase sharing proregion sequence homology with human β-tryptase (Wolters et al. 2001). Indeed, in vitro studies indicate that pro-β-tryptase undergoes auto-cleavage, resulting in a two amino acid residue activation dipeptide that is subsequently removed by cathepsin C catalytic activity (Sakai et al. 1996). Nonetheless, these results indicate that cathepsin C may not be essential for activation of all mouse mast cell tryptases (Sheth et al. 2003, Wolters et al. 2001).

Interestingly, cathepsin C-dependent activation cascades induce multiple pathways that may be important for cancer development, namely those culminating in the conversion of pro-MMP-9 and pro-MMP-2 to their mature forms, thus greatly amplifying MMP bioactivity (Fig. 9.1). Indeed, following activation by cathepsin C, mMCP-4, the major source of stored chymotrypsin-like activity in mouse peritoneum and skin (Tchougounova et al. 2003), further activates pro-MMP-2 and pro-MMP-9 (Tchougounova et al. 2005). In addition, neutrophil-derived NE, cathepsin G, and proteinase 3 have also been found to regulate MMP-2 activation (Shamamian et al. 2001). While these proteases are unable to process soluble pro-MMP-2, recent studies indicate that binding of pro-MMP-2 to the membrane-tethered MMP-14 induces conformational change, rendering a cleavage site within pro-MMP-2 prodomain region available for cleavage by neutrophil-derived serine proteases (Shamamian et al. 2001). These observations raise an interesting point – that being redundancy as a common theme among proteolytic cascades.

In addition to the role of cathepsins in maintaining tissue homeostasis and regulating diverse enzymatic activities, recent studies have revealed their involvement as mediators of inflammation. While mice harboring homozygous deletions in the cathepsin C gene exhibit normal neutrophil chemotactic responses to thioglycollate, mice were resistant to experimental acute arthritis and displayed altered neutrophil recruitment in response to zymosan and immune complexes, a defective response rescued by administration of a neutrophil chemoattractant (Adkison et al. 2002). Likewise, using the air pouch model of inflammation, the number of infiltrating neutrophils was significantly attenuated in mice deficient in both NE and cathepsin G($NE^{-/-} \times CG^{-/-}$; Adkison et al. 2002). Notably, a significant decrease in local levels of chemokines, including tumor necrosis factor (TNF)-α and interleukin-1β (IL-1β), was observed in air pouch microenvironment of cathepsin C-deficient mice as well as $NE^{-/-} \times CG^{-/-}$ mice. Moreover, injection of IL-8, a neutrophil-specific chemokine, restored infiltration of neutrophils into air pouches of cathepsin C-deficient mice (Adkison et al. 2002). These results provide novel insights into the role of neutrophil-derived proteases in inflammation, in addition to their ability to modulate ECM components, by regulating local levels of chemoattractants at sites of inflammation (Adkison et al. 2002).

Using de novo carcinogenesis models in cathepsin-deficient tumor-prone mice has implicated roles for individual cathepsins in distinct tumorigenesis processes. Profiling of differential expression and activity of cathepsins in normal, premalignant, and malignant islets of RIP1-Tag2 mice revealed a complex cascade of sequential cathepsin expression correlating with tumor development (Gocheva et al. 2006, Joyce et al. 2004). Taking a genetic approach, Joyce and colleagues eliminated single cathepsin genes and assessed their unique roles to islet carcinogenesis and found that while tumor-associated angiogenesis was significantly reduced in the absence of cathepsin B or S, genetic elimination of cathepsin B or L instead attenuated tumor cell proliferation and decreased tumor volume, while absence of cathepsin C was without consequence (Gocheva et al. 2006). Similar studies by Peters and colleagues found that during mammary carcinogenesis in MMTV-PymT transgenic mice (Guy et al. 1992), absence of cathepsin B emanating from macrophages significantly limited primary tumor development as well as pulmonary metastasis formation (Vasiljeva et al. 2006). Interestingly, whereas cathepsin B was found to be a significant protumor regulator of pancreatic and mammary carcinogenesis, cathepsin B does not appear to be functionally significant during skin carcinogenesis (Junankar and Coussens, unpublished observations). Moreover, whereas absence of cathepsin C during islet carcinogenesis is without consquence, its absence during squamous carcinoma development in K14-HPV16 transgenic mice is profound (Junankar and Coussens, unpublished observations). During murine skin carcinogenesis, like in humans afflicted with loss of mutations in the cathepsin C gene (de Haar et al. 2004, Frezzini et al. 2004, Hewitt et al. 2004, Noack et al. 2004), myeloid cells fail to infiltrate damaged tissue (Adkison et al. 2002, Pham et al. 2004, Pham and Ley 1999); thus, protumor programs (angiogenesis, matrix remodeling) regulated by inflammation, fail to be activated.

Au4

Au5

In summary, profiling cysteine cathepsin activities in various mouse models of multistage cancer revealed interconnecting protease cascades that are initiated by a common upstream protease activator, cathepsin C, and culminating in amplification of enzymatic activity of "terminal" proteases such as MMP-9 (Fig. 9.2). Importantly, these studies identified individual cathepsins that play differential roles in specific cancers emanating from multiple organ sites, and thus affirming the significance of organ- and tumor type-specific regulation of protease bioactivities.

Functional Role for Amplifying Protease Activities

Collectively, a vast body of literature indicates that lysosomal and pericellular proteases act as cofactors for cancer development. Rather than functioning individually, each protease can be regarded as a "signaling molecule" that exerts its effects as part of a proteolytic pathway, where proteases potentially interact and activate other proteases in a cascade-like manner, thus triggering formation of interconnecting protease circuits that form the so-called "protease web" (Overall and Kleifeld 2006). Notably, these linear protease circuits converge, leading to amplification of net proteolytic activity of enzymes like MMP-9 within tissues and consequently enhancing development of pathological conditions (Fig. 9.2). Alternatively, by using separate protease circuits, the same tissue may achieve comparable net substrate activation under different pathological settings. These observations shed light on the importance of characterizing how a tissue responds under distinct circumstances by differentially activating specific proteolytic pathways. Nonetheless, in an attempt to understand the functional roles of proteases in cancer development, it is necessary to further view proteolysis as a "system" by incorporating all the elements of the protease web, including not only proteases but inhibitors, cofactors, cleaved substrates, and receptors as well.

Perturbations of specific protease circuits may profoundly influence the normal balance of enzymatic activities, affecting the net proteolysis as a whole and leading to development of distinct pathological conditions. As an example, loss-of-function mutations in the cathepsin C gene are associated with the autosomal recessive disease Papillon-Lefevre syndrome (PLS) and Haim-Munk syndrome, characterized by severe periodontal inflammation and skin lesions, as well as increased susceptibility to infections (Hart et al. 2000, Noack et al. 2004). These symptoms may be attributed to a severe reduction in the levels and activities of multiple neutrophil granule-associated serine proteases, including NE, cathepsin G, proteinase 3, and mast cell chymases. Thus, to understand the underlying pathology of PLS, it is important to first define physiological substrates for cathepsin C – that is if we step back and analyze the consequence of cathepsin C activation within the context of a proteolytic pathway, such studies may reveal the mechanisms by which cathepsin C-deficiency mediates disease processes. More importantly, protease biology is a dynamic field, where potential activators and substrates are continuously identified. For example, using a membrane permeable inhibitor of cathepsins,

intracellular levels of collagen degradation were reduced in human breast carcinoma cells (Sameni et al. 2000), indicating a novel mechanism by which tumor cells degrade collagen intracellularly within lysosomes, as opposed to the traditional role of collagenolytic MMPs as the ultimate extracellular effectors of ECM catabolism (Mott and Werb 2004). Such mechanisms include identification of new molecules that link lysosomal cathepsin B and cell surface-bound pro-uPA (Sameni et al. 2000), such as uPAR-associated protein (uPARAP; *see* Chap. 13 by Hillig, Engelholm and Behrendt, this volume; Behrendt et al. (2000); Fig. 9.2]. Following formation of a ternary complex with pro-uPA and uPAR, uPARAP is internalized along with bound collagen to endosomal compartments where cathepsins mediate collagen degradation (Curino et al. 2005, Mohamed and Sloane 2006). These studies indicate that strategies aimed at inhibiting proteases should be carefully designed, given the possibility that pharmacological inhibition of MMP pericellular activities may be counteracted with increased cathepsin-dependent intracellular catabolism of ECM components. Taken together, the net proteolytic activity of neoplastic tissues can no longer be understood without taking into consideration the cascade of protease activities and their potential interconnections – that is the flow of information within the protease network as a whole.

Targeting Proteases or Novel Use of Proteases Within the Tumor Microenvironment?

Important challenges for the future include characterization of the "cancer degradome" at the protease and substrate levels. Which proteases are differentially active in specific tumor types? What substrates do they activate and what interconnections and networks can they potentially form? Alternatively, it is clear that disturbance of individual proteases induces alterations in multiple levels of protease cascades. Should future studies focus on combinatorial approaches targeting multiple proteases as opposed to individual proteases? Answering this question is illustrated by recent studies using direct intratumoral injections of interfering RNAs (RNAi) that demonstrated simultaneous down-regulation of cathepsin B and MMP-9 (Lakka et al. 2004) as well as MMP-9 and uPAR (Lakka et al. 2005, Lakka et al. 2003) resulting in regression of preestablished tumors more effectively than targeting either protease alone. Nonetheless, although these approaches are promising, future studies should take into consideration the possibility that alternative pathways may be activated to compensate for complete loss of one enzymatic activity. For instance, following genetic elimination of cathepsin B, tumor cells have been found to induce cathepsin X expression, resulting in a partial compensation for loss of cathepsin B-mediated effects (Vasiljeva et al. 2006).

In contrast, will future strategies take advantage of unique characteristics limited to specific protease pathways? Proteases may be restricted to certain regions of the cell, such as proteases that are tethered to cell surfaces, including uPA, thus directing proteolysis to discrete focal areas versus intracellular proteases within

lysosomes, such as cathepsins. Recent studies exploiting unique localization of specific protease pathways to direct activation of nontoxic "prodrugs," such as doxorubicin (Dox), selectively to tumor sites [*see* also Chap. 39 by Fields and Chap. 40 by Gill and Loadman, this volume; Devy et al. (2004)]. Here, incorporation of a tripeptide specifier recognized exclusively by tumor-associated plasmin initiated release of free Dox from its prodrug form selectively in the vicinity of tumor cells, given that the systemic presence of physiological plasmin inhibitors, including α_2-antiplasmin and α_2-macroglobulin, limits Dox activation within the circulation (Devy et al. 2004). Using such approaches, Dox has been shown to exert its cytotoxic and/or cytostatic effects locally while restricting cardiotoxicity, which may limit Dox dosage intake. Similar strategies have been used to design prodrugs that are activated in tumor cells over-expressing selective protease pathways, including cathepsin B. Indeed, Panchal et al. (1996) engineered α-hemolysin, which once activated by tumor cells expressing high levels of cathepsin B, induces selective apoptosis of tumor cells, thus reducing side effects associated with the use of this drug.

Furthermore, recent advances in novel anticancer therapies took advantage of selective expression of the antiapoptotic protein survivin in malignant ovarian cells to specifically activate expression of proapoptotic proteases, such as cytotoxic T lymphocyte-derived granzyme B (Caldas et al. 2006). Driven by the survivin promoter, active granzyme B not only reduced tumor incidence and size of xenografted human ovarian carcinoma in nude mice but also prevented metastatic spread (Caldas et al. 2006). Such approaches illustrate the utility of proteases that are normally employed by immune cells to eliminate tumor cells in designing future therapeutics. Taken together, to advance the understanding of individual proteases and delineate their physiological as well as pathological roles, the complexity by which proteases interact must be taken into account as opposed to merely investigating individual proteases.

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Appendix B

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Development of an optimized activatable MMP-14 targeted SPECT imaging probe

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ABSTRACT

Matrix metalloproteinase-14 (MT1-MMP or MMP-14) is a membrane-associated protease implicated in a variety of tissue remodeling processes and a molecular hallmark of select metastatic cancers. The ability to detect MMP-14 in vivo would be useful in studying its role in pathologic processes and may potentially serve as a guide for the development of targeted molecular therapies. Four MMP-14 specific probes containing a positively charged cell penetrating peptide (CPP) d-arginine octamer (r_8) linked with a MMP-14 peptide substrate and attenuating sequences with glutamate (8e, 4e) or glutamate-glycine (4eg and 4egg) repeating units were modeled using an AMBER force field method. The probe with 4egg attenuating sequence exhibited the highest CPP/attenuator interaction, predicting minimized cellular uptake until cleaved. The in vitro MMP-14-mediated cleavage studies using the human recombinant MMP-14 catalytic domain revealed an enhanced cleavage rate that directly correlated with the linearity of the embedded peptide substrate sequence. Successful cleavage and uptake of a technetium-99m labeled version of the optimal probe was demonstrated in MMP-14 transfected human breast cancer cells. Two-fold reduction of cellular uptake was found in the presence of a broad spectrum MMP inhibitor. The combination of computational chemistry, parallel synthesis and biochemical screening, therefore, shows promise as a set of tools for developing new radiolabeled probes that are sensitive to protease activity.

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1. Introduction

Tumor cells employ a host of biochemical mechanisms in order to invade and metastasize. Many of these mechanisms are thought, in part, to involve proteases associated with cell membrane and extracellular matrix (ECM) molecules that are posited to initiate pro-angiogenic signaling cascades. Among cancer-associated proteases, matrix metalloproteases (MMPs), a class of zinc-dependent proteolytic enzymes, have been postulated to be used by cancer cells to dissolve ECM during neoplastic progression.[1] In addition, numerous studies have documented a positive correlation between certain MMP expression levels and poor outcome in cancer patients.[2] The importance of MMPs in tumor progression not only has guided the development of MMP inhibitors for therapy, it has also received particular attention as imaging target utilizing methods to detect tumor-associated proteolytic activity in vivo.[3–7]

The family of human MMPs contains 16 secreted and 7 membrane-tethered enzymes.[8] A subclass of the membrane-anchored proteinases, termed membrane type (MT) MMPs, plays dominant roles in controlling cancer cell behavior.[9,10] In particular, the up-regulation of the membrane-associated collagenase MMP-14 (MT1-MMP) correlates to the invasiveness of many different tumor types.[2] MMP-14 not only promotes tumor growth through induction of angiogenesis and proteolysis of ECM, but it also acts as a critical regulatory switch in the activation of MMP-2 proenzyme.[11] Clinical studies revealed that the expression of MMP-14 is associated with poor prognosis in patients with advanced neuroblastoma,[12] small cell lung cancer (SCLC),[13] tongue squamous cell carcinoma,[14] head and neck carcinoma,[15] bladder, and ovarian cancer.[16,17] MMP-14 has been detected in tumor cells and

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adjacent stromal cells in a variety of human tumors including breast.[9] Consequently, MMP-14 overexpression holds great promise as an early biomarker for invasive cancers.

In the past, several in vivo optical imaging probes targeting various MMPs have been reported; the most successful of these efforts have been directed against MMP-2, -7, and -9.[18–23] Attempts to image MMP activities by non-optical modalities (e.g., positron emission tomography (PET) or single photon emission computed tomography (SPECT)) using labeled substrates or inhibitors, however, have met with limited success in vivo, in part due to the poor specificity and in vivo stability of the radiolabeled probes.[24–31] Our motivation, therefore, was to develop a sensitive nuclear probe for MMP-14 activities for early cancer detection. The success of such a probe would represent a significant advancement in preclinical and clinical imaging as it would be a tool able to locate and track the molecular evolution of malignant tissues for use in drug development.

A number of protease imaging strategies have been described previously. One particular class of probes comprising an 'activatable' delivery mechanism has been developed by a number of research groups.[21,23,32] These probes share a core structure consisting of a poly-D-arginine cell penetrating peptide (CPP) that is covalently tethered to a negatively charged attenuating peptide sequence through a proteolysis sensitive peptide (Fig. 1). The intact probe is believed to be prohibited from crossing the cell membrane due to the electrostatic interaction between the positively charged arginine and the tethered intramolecular negatively charged attenuator (I), but other mechanisms may also contribute. However, proteolytic cleavage (II) separates the polyarginine sequence from the negatively charged domain, thereby triggering uptake of the CPP (III). Protease-rich tissues may be imaged by tagging an imaging reporter group, such as a fluorophore, gadolinium chelate or radionuclide, to the CPP.[32] As a result, the number of protease cleavage events may be correlated to the CPP concentration, and its associated tag, within targeted cells. Using agents directed against MMP-2/9 and -7, Rao and co-workers have selectively tagged cultured fibrosarcoma cells (HT-1080) with quantum dots,[23] while Tsien and co-workers successfully imaged cancers rich in MMP-2 and -9 in murine xenografts, using optical and magnetic resonance techniques.[21]

This general strategy is attractive because the catalytic processing of more than one probe by each enzyme provides a robust mechanism for signal amplification. For the purpose of MMP-14 imaging, this is a particularly important point if one seeks to detect protease activity prior to the maturation of secondary downstream proteases, for example, MMP-2 and -9.

The development of an 'activatable' SPECT imaging probe specific for MMP-14 is reported herein. The probe design was undertaken realizing that attaching a large metal chelate for nuclear imaging may alter the topology of the MMP-14 selective peptide sequence and adversely affect the cleavage rate as well as attenuation characteristics of the basic probe platform. Therefore the combination of molecular modeling, parallel synthesis and bioassay screens were effectively utilized to optimize the imaging probe construct for MMP-14 activities. This work sheds new light on the intramolecular quenching and activation mechanisms of this class of imaging peptides, and demonstrates the value of computational chemistry relative to imaging probe development.

2. Results and discussion

2.1. MMP-14 probe design and modular components

The general MMP-14 probe, shown in Figure 2, is a modular design comprised of three components: (A) a positively charged d-arginine octamer (r_8) cell penetrating peptide (CPP) attached with single amino acid chelate (SAAC) for technetium-99m; (B) a MMP-14 specific cleavable substrate (SGRIGF↓LRTA) and (C) a negatively charged attenuation sequence. The seminal component for designing an effective MMP-14 probe was a suitable cleavable peptide substrate. A known MMP-14 substrate (SGRIGF↓LRTA) was incorporated into our constructs to maximize the selectivity of the probe for MMP-14-rich tissues. This sequence was originally described by Smith and co-workers, who employed phage display technology to map the substrate specificity of matrix metalloproteinases.[33] In an enzyme panel study, this peptide sequence is preferentially cleaved by MMP-14 with a k_{cat}/K_m value of 777,200 M^{-1}/s, while the cleavage by the related MMP-9 is much less efficient, with a k_{cat}/K_m of 20,000 M^{-1}/s.

Although poly-D-arginine sequences with chain lengths of 7–14 residues have been widely employed as cell membrane penetrating transporters[34] in probe constructs, there has been little consensus over the degree of attenuation necessary for effective proteolytic cleavage and reducing cell penetration. In designing the MMP-14 probe, the attenuation module was optimized considering: (1) the number of negatively charged residues for effective attenuation; (2) the structural impact on the cleavable peptide sequence

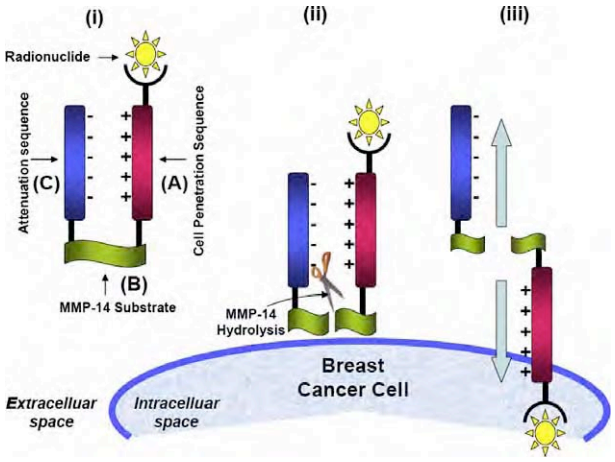

Figure 1. Outline of the probe structure and mechanism. The quenched probe (I) is able to freely circulate in vivo, until it encounters its protease target. Cleavage of the probe at a defined point by MMP-14 (II) releases a cell penetrating peptide, which can then translocate its radionuclide cargo across the target cell membrane (III). After uncleaved quenched probe is washed away, the internalized radioactivity can be imaged by SPECT.

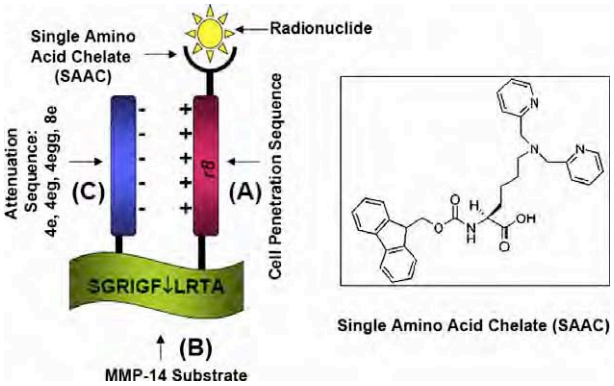

Figure 2. The general modular design of the MMP-14 probe comprising: (A) positively charged arginine octamer (r8) cell penetrating peptide with the single amino acid chelate (SAAC) coordinated with a radionuclide; (B) MMP-14 peptide substrate (SGRIGF↓LRTA) and (C) variation of D-glutamate attenuation sequences (4e, 4eg 4egg and 8e).

and (3) the ability to release the cleaved probe for cellular uptake. Attenuation of D-arginine octamer (r_8) CPP was favorably achieved by inserting the optimal number of D-glutamate residues in the attenuation domain, such that the cleavable peptide sequence was present in a roughly linear conformation and available for docking to the MMP-14 enzyme. A small panel of attenuation sequences, including four D-glutamates (4e), four D-glutamate-glycine repeats (4eg), four D-glutamate-glycine-glycine repeats (4egg) and eight contiguous D-glutamates (8e), were evaluated using computer modeling. The D version of the amino acid residues were chosen to prevent non-specific in vivo proteolysis. Based on prior experience, the choice of four D-glutamates appropriately spaced in three of the four attenuator sequences adequately attenuates the r_8 peptide.[32]

Finally, in order to conduct radionuclide imaging by SPECT, it is necessary to selectively label the peptide probe with a suitable isotope. To this end, the single amino acid chelate (SAAC) technology, previously developed by Stephenson et al.,[35,36] has been utilized. The SAAC is a Nε-bis(pyridylmethyl)-lysine derivative that may be site-selectively incorporated into synthetic peptides using standard Fmoc chemistry. The resulting compounds carry an N-3 chelator that form stable tricarbonyl complexes with technetium and rhenium. Technetium-99m, a gamma ray emitter, has emissions of sufficient energy (140 keV) to penetrate the human body, but not too energetic to pass through the SPECT detector material. It is considered to be a near-ideal isotope for SPECT imaging, in part due to its short half-life (6 h), its gamma emission energy and its availability from a commercial generator system. Based on these characteristics, technetium-99m has been chosen in the current probe design to visualize cell penetration upon proteolysis.

2.2. Computational chemistry

Holding the CPP and MMP-14 peptide regions constant and using the cysteine–cysteine disulfide bond as a linker between modules, the overall structure with four different attenuation sequences containing D-glutamate residues (4e, 4eg , 4egg and 8e) was computed. The extent of charge-complementation was calculated between the CPP and the attenuation sequence by examining the number of arginine residues that were in contact with the glutamate residues. The average end-to-end backbone length of the cleaved substrate was measured to quantify the extent of exposed substrate, in an available extended conformation, for effective enzyme pocket binding for subsequent cleavage by the MMP-14. Based on these calculations, the dominant conformation for each probe, shown in Figure 3, was determined.

These dominant structures reveal that the 4e probe has minimum interaction between the CPP (blue) and the attenuation sequence (red). Structural calculations confirm the overlap finding (Fig. 4A) with the 4e probe possessing the least CPP-attenuator complementation at an average of 4.3 ± 1.5 arginine residues in contact with D-glutamate while contact in the other three probes range from 5.2 ± 1.6 to 6.4 ± 1.2 residues. Here, the standard deviation reported does not indicate the standard error in the measurement. Instead, it corresponds to the root mean square fluctuation of the measurement within the computed peptide conformational ensemble, that is due to the natural conformational fluctuations experienced by the peptide at ambient conditions. These data suggest that the 4e motif may not be as effective in attenuating the translocation of the CPP prior to proteolytic cleavage. As the overall probe structure was examined,

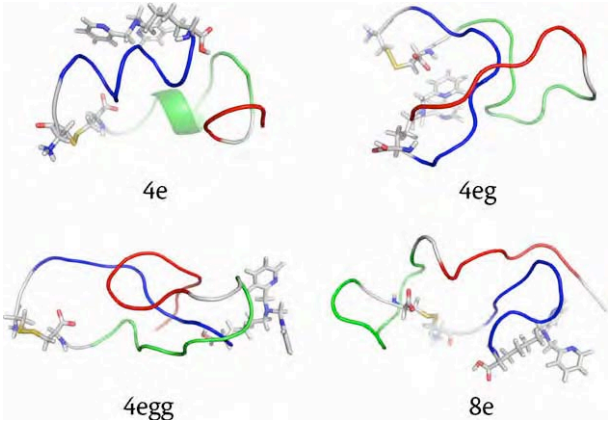

Figure 3. Structures of MMP-14 probes determined from computer simulations using the zipping and assembly method. In each structure, the backbone is shown in a cartoon representation, colored as follows: blue = CCP, green = MMP-14 specific substrate region, and red = attenuating domain. Both the Single Amino Acid Chelate (SAAC) residue and the cysteine–cysteine linker unit are shown in bond representation.

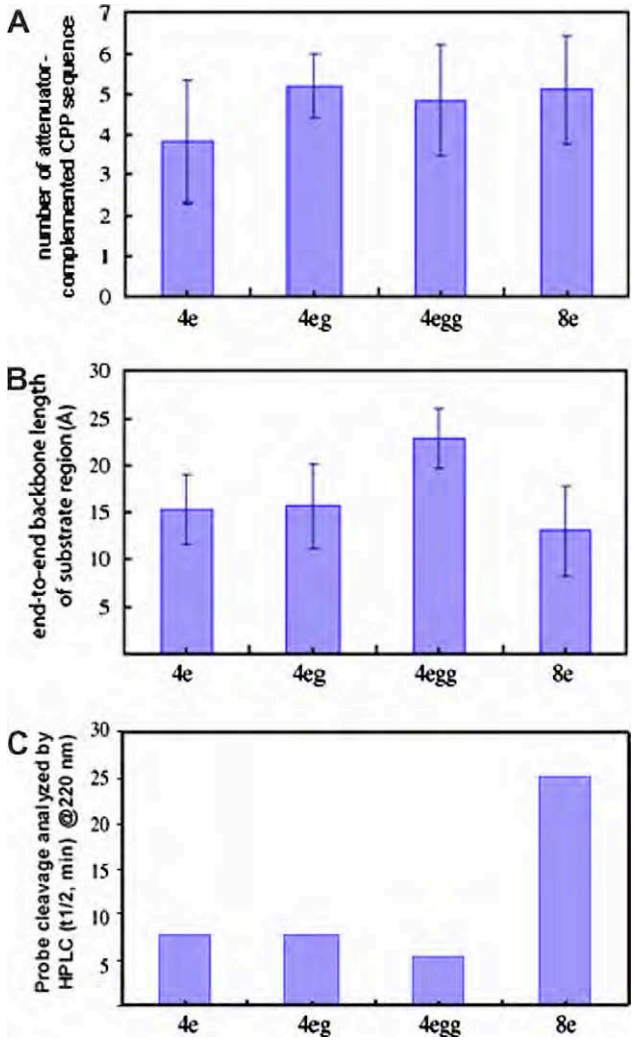

Figure 4. Calculated structural metrics for the four peptides and side-by-side comparison to experimental cleavage data. (A) The computed average (over the structural ensemble) number of arginine residues in the CPP that are in contact with at least one glutamate residue in the attenuating region. (B) The computed average end-to-end distance (Å) of the MMP-14 substrate region; larger values indicate that the substrate is closer to a linear conformation. The error bars show the average fluctuations over the conformational ensemble. (C) Parallel cleavage studies; half-life ($t1/2$, in min) of each probe measured in the presence of MMP-14 by HPLC peak integrations at an absorbance of 220 nm.

the CPP-attenuator complementation was also found to influence the conformation of the MMP-14 substrate region, potentially altering the accessibility for proteolytic cleavage. As the end-to-end distance of the MMP-14 substrate is a direct measure of peptide folding, it also indirectly predicts the availability of the peptide substrate for cleavage. The calculations show (Fig. 4B) that the 4egg probe best adapts the β-strand conformation[33] required for rapid MMP-14 cleavage, exhibiting the longest end-to-end distance of 21.62 ± 4.3 Å. The 4e (16.9 ± 2.9 Å) and 4eg (17.1 ± 4.3 Å) sequences show a similar degree of folding, whereas the 8e probe is the most distorted from linearity (15.0 ± 2.9 Å). This modeling study predicts that the 4egg probe has adequate CPP attenuation in the absence of MMP-14 and allows a β-strand type conformation for the substrate portion of the probe to bind and interact with the MMP-14 catalytic domain.

2.3. Parallel probe synthesis

A modular approach to the panel design was adapted to accelerate the synthesis of the peptide probes. Each probe was retrosynthetically divided into two fragments of comparable size. One peptide fragment common to all of the probes contains the CPP and the SAAC with an additional cysteine and glycine appended to the N-terminus. Four variable fragments, containing the SGRIGF↓LRTA sequence and the attenuators, were also appended with glycine and cysteine at the C-terminus. This allowed all five fragments (1 conserved, 4 specific to each probe) to be synthesized in parallel and to be subsequently assembled into the probe construct (Fig. 2) through simple disulfide chemistry.

Typical synthesis of the various probes yielded the desired constructs along with two homo-dimers. The isolated probes were characterized by analytical HPLC, MALDI-MS and ^{1}H NMR. As expected, the desired MMP-14 probes exhibited retention times between the homo-dimer peaks. MALDI-MS analysis showed the expected molecular ion peak along with the characteristic fragmentation pattern produced by homolytic cleavage of the disulfide bond and loss of pyridylmethylene radicals from the SAAC (Fig. 5).

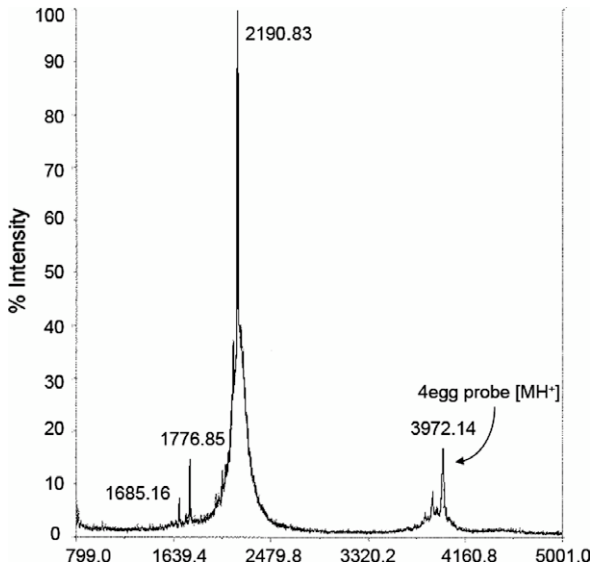

Figure 5. A sample MALDI-MS of the 4egg probe. The presence of the molecular ion peak (3972 amu, $[MH]^+$), peptide fragments corresponding to disulfide scission (2191 and 1777 amu), and a fragment corresponding to loss of a pyridylmethylene radical from the SAAC-containing parent peptide (1685 amu = 1777–92).

2.4. Proteolytic cleavage study with purified MMP-14 enzyme

A sample of each probe was subjected to enzymatic digestion by MMP-14, followed by HPLC analysis. As expected, each probe generated two clear cleavage products upon proteolysis, indicating that the cleavage event was largely restricted to a single cleavage site. The cleavage rate was characterized by the probe half-lives and initial cleavage velocities based on the first-order kinetics. Significant differences in cleavage rates between the probes were observed. As shown in Figure 4C, the increasing cleavage rate rank order was 8e < 4e ≈ 4eg < 4egg. Regression analyses confirm that all eight data sets are strongly linear, with r^2 values ranging from 0.90 to 0.99. These data correlate well with the end-to-end distance measures derived from the molecular modeling studies where the 8e probe MMP-14 substrate was most distorted from linearity, the 4e and 4eg probes were similar length yet longer than the 8e probe, while the 4egg probe possessed the longest end-to-end distance.

As previously suggested by Smith and co-workers,[33] the SGRIGF↓LRTA cleavage sequence is believed to possess the β-strand conformation for efficient binding to MMP-14 and to facilitate the subsequent catalytic reaction. In the present study, protein structure related to the interaction of the attenuator with the CPP changes the embedded MMP-14 substrate conformation directly affecting the cleavage rate. The molecular modeling of this small library of structures efficiently predicts the cleavage rate as a function of substrate linearity. Thus, the attenuation sequence may be chosen for effective attenuation of the CPP and presentation of an optimized structural conformation for rapid enzymatic cleavage.

2.5. Radiochemistry with ^{99m}Tc

The carboxylate terminal of the r_8 CPP was linked to a single amino acid chelate (SAAC) through a peptide bond. Based on our results in molecular modeling and MMP-14 cleavage study, the optimal probe, 4egg, was chosen to demonstrate labeling efficiency. The 4egg probe was successfully labeled with technetium-99m through the $[^{99m}Tc(CO)_3(OH_2)_3]^+$ intermediate. The final ^{99m}Tc- labeled probe was isolated by HPLC with 97% radiopurity and a radiochemical yield greater than 90%.

The SAAC technology, developed by Zubieta and colleagues, has shown great promise in small molecule radiopharmaceutical applications.[35,37,38] The SAAC, a lysine based residue with a built-in chelating system for binding rhenium or technetium tricarbonyl, was chosen over direct peptide labeling techniques to avoid non-specific binding of the label on the backbone structure. The SAAC also allows labeling of the molecule as the final step. Labeling an intermediate that is subsequently reacted with the final molecule may reduce yield, while bulky chelating molecules or bifunctional chelates used in direct labeling may significantly effect biodistribution of the labeled compound.[39] For instance, Polyakov and colleagues have synthesized a Tat CPP incorporating a peptide-based motif (epsilon-KGC) that provides an N_3S donor core for chelating technetium and rhenium. They have successfully demonstrated its uptake in Jurkat cells after labeling with oxotechnetium(V) or oxorhenium(V).[40] Although this approach offers labeling specificity, each donor core requires three additional residues. The current probe design and prototype, on the other hand, utilizes a single residue for each technetium or rhenium tag. In addition, this strategy for radiolabeling provides control over the isotope delivery system thereby permitting the MMP-14 probe to be labeled with technetium-99m (a pure gamma emitter) or rhenium-186 (a beta and gamma emitter) that will not only allow early detection of invasive cancer, but it will also provide a common platform for radiotherapy.

2.6. In vitro uptake of the ^{99m}Tc labeled probe

Human epithelial breast cancer (MDA-MB-231) cells transfected with MMP-14 cDNA to overexpress MMP-14[41] were used to evaluate the in vitro uptake of the labeled 4egg probe. The effect of probe activation through cleavage by MMP-14 at cell surface was determined by treatment of the transfected MDA-MB-231 cells with and without the potent broad-based MMP inhibitor, GM1489. Free ^{99m}Tc-tricarbonyl complex was compared as a null control. All cell studies were carried out in triplicate. After the treatment with the labeled 4egg probe, the adhesive cells were carefully washed with DMEM, before being released from the plate with trypsin. The probe content within each media and cell fraction was then quantified by a gamma counter (Wizard 3, Perkin–Elmer, CT). Each data point represents the fraction of the probe uptake by cells quantified by gamma count before and after incubation and washings of cells (approximately 10^5 cells per well). As shown in Figure 6, there is a clear difference in the cellular uptake of probe in the presence and absence of the MMP inhibitor. There was little or no uptake of the free $[Tc(CO)_3]^+$ by the cells. Despite the larger variation in cells without inhibitor (may due to cell handling), the average uptake of the 4egg probe was two times greater in cells without inhibitor compared to those with inhibitor. This demonstrates the successful cleavage and increased uptake of the activated probe into MMP-14 expressing cells. The residual 10–15% uptake in cells treated with MMP inhibitor indicates that there may be incomplete inhibition of MMP-14 activity by the inhibitor, or a basal level of probe leakage attributable to incomplete attenuation of the CPP. The negative results from $[^{99m}Tc(CO)_3]^+$ suggests that (a) there is no non-specific uptake of free $[^{99m}Tc(CO)_3]^+$ and (b) the positive uptake of the probe is the result of the cleaved probe rather than leakage of free $[^{99m}Tc(CO)_3]^+$ into the transfected cells. Attempts have been made to use MDA-MB-231 cells without MMP-14 transfection as a negative control. However, uptake of the 4egg probe was also observed. This may due to cleavage reactions by combination of other MMPs at basal level.[33] Nevertheless, the reduction in uptake by transfected cells in the presence of inhibitor suggests that the 4egg probe possesses MMP-14 specificity, and it warrants further efforts in probe development.

3. Conclusion

The unique application of molecular modeling in optimization of the MMP-14 probe construct has been demonstrated. Modeling calculations were able to provide useful information on the attenuation properties of four different sequences against the r_8 CPP and the length of the MMP substrate that is directly related to cleavage rate. Both of attenuation and cleavage must be maximized in the optimal probe. Although three out of four probes studied exhibited good CPP attenuation, the CPP/attenuator interaction contributed to structural modifications of the MMP-14 substrate altering the cleavage rate. The probe with the longest end-to-end substrate length, 4egg, exhibited the fastest cleavage rate. Evaluation of the 4egg probe in cells expressing MMP-14 demonstrated the specific activation of the probe and subsequent accumulation of the label in the cell. The value of computational modeling to imaging probe design has been demonstrated and this methodology will be incorporated into future tumor imaging probe platform development.

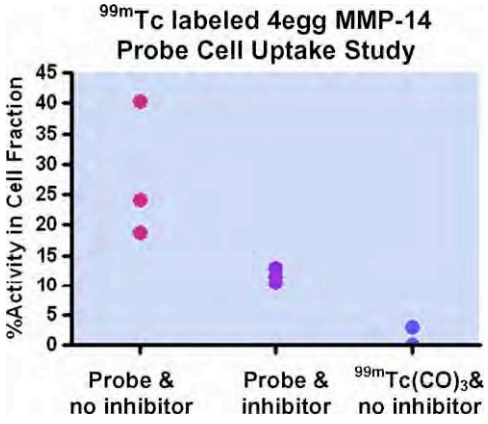

Figure 6. In vitro uptake of the 4egg probe in MMP-14 expressing MDA-MB-231 cells with and without metalloproteinase inhibitor. $[Tc(CO)_3]^+$ was used as a negative control.

4. Experimental

4.1. Molecular modeling

Computer simulations were used to predict the solution structures of the peptide probes employing the AMBER96 force field[42] with the implicit solvation model of Onufriev, Bashford, and Case.[43] This force field was validated in a separate study and shown to give accurate structures for peptides with both alpha and beta motifs.[44] For the non-canonical chelate residue, the program 'antechamber' in the AMBER package was used to determine atomic charges and estimate force field constants. The SAAC chelate was included in the molecule without the radiometal for the simulations.

Sampling was performed using the zipping and assembly (Z&A) methodology developed by the Dill research group.[45] Z&A works by sampling peptides and proteins according to a putative folding mechanism:[46] peptide pieces along the chain independently first form small bits of structure, which then either nucleate additional structure locally by reeling in nearby sections of chain (zipping), or join together with other such structured peptide pieces to form larger units (assembly). This mechanism is implemented in simulation by first breaking a peptide into small fragments, which are simulated separately, and then by growing these fragments by the addition of new residues and other fragments, followed in each case by further simulation. Sampling at each stage is performed using replica exchange molecular dynamics.[47]

For each of the probe candidates, the overall chain was broken into the three fragments corresponding to the attenuator, substrate, and translocation domains. These were simulated separately in replica exchange for 10 ns, and conformations from each fragment from the final 1 ns were clustered into a maximum of 10 structures using a modified k-means algorithm. Subsequently, clustered conformations from the substrate and translocation domains were combined (in all permutations), and used in a second 10 ns replica exchange simulation, followed by additional clustering. Next, the attenuating domain structures were added, followed by 10 ns of sampling, and finally, the terminal chelate residue was added, with 30 ns of replica exchange as the last sampling step. The clustered conformations from the last 5 ns of the 30 ns run (along with their populations) were taken as the final conformational ensemble. In total, each peptide required about 300 aggregate CPU days worth of simulation time on Xeon 2.4 GHz processors.

4.2. MMP-14 probe synthesis

Uronium coupling agents (2-(1*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate or tetrafluoroborate; 'HATU' and 'TBTU') and Fmoc-protected amino acids were purchased from Novabiochem, Inc. The Nα-Fmoc-Nε-bis(pyridylmethyl)-lysine was received from Molecular Insight Pharmaceuticals, Inc. or synthesized as previously described.[42] Solvents, including dichloromethane and dimethylformamide, were pur-

chased from VWR Scientific. All other reagents were obtained from Sigma–Aldrich Chemical Company, and used without further purification.

Peptide intermediates were synthesized on a Protein Technologies, Inc. Prelude peptide synthesizer according to standard Fmoc chemistry protocols. In some cases, Fmoc deproptections were effected using 6% piperazine in dimethylformamide,[48] as opposed to the more traditional 20% piperidine in DMF. All sequences were cleaved from the resin and deprotected using 92:3:3:1:1 trifluoroacetic acid/triisoproylsilane/water/thioanisole/1-naphthol for 2 h. Crude peptides were obtained by triturating the cleavage solution with ice-cold ether, followed by centrifugation. All peptide starting materials were purified by semi-preparative, reverse-phase HPLC (Jupiter C12 column from Phenomenex; water-acetonitrile gradient, 95% water to 5% water; 0.1% trifluoroacetic acid added to the mobile phase to maintain a pH ~2.)

The completed probes were assembled as heterodimeric disulfides. Cysteine oxidation was conducted using the following general procedure: Ac-CGrrrrrrrrr(SAAC)-$CONH_2$ (~1.8 mg.; 1 μmol) and a suitable attenuator-MMP-14 substrate peptide (~3 to 4 mg., according to the mass of the attenuator sequence; 1 μmol) were dissolved in 0.75 mL of 5:3:2 water/0.2 M borate buffer, pH 8/methanol. The peptides were pre-reduced by addition of 50 μL of 1 mg/mL tris-(2-carboxyethyl)phosphine hydrochloride, dissolved in the same reaction buffer. After 15 min, the pH was checked to ensure the reaction mixture remained above 8; then, 100 μL of DMSO was added with swirling, followed 5 min later by 5 mg. of potassium hexacyanoferrate (III). The resulting yellow solution was covered with foil, and allowed to stand overnight. Following acidification with 50 μL of acetic acid, the reaction mixture was diluted to ca. 1 mL with deionized water, and purified by semi-preparative HPLC (Jupiter C12 column from Phenomenex; water–acetonitrile gradient, 95% water to 5% water; 0.1% trifluoroacetic acid added to the mobile phase to maintain a pH ~2.) Typically, three major peaks were observed at 220 nm (two peaks at 260 nm), corresponding to the desired heterodimer and homodimerized reactants. The reactant homodimers could be recovered and reductively recycled for use in future reactions.

4.3. MMP-14 cleavage kinetics

Approximately equimolar samples of each unlabeled probe were prepared in MMP-14 hydrolysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 5 mM $CaCl_2$, 0.025% Brij-35)[49] as verified by HPLC analysis and peak integration at 260 nm. Each of these peptide stock solutions (0.8 mL) was transferred to a separate microcentrifuge tube, and equilibrated at 37 °C for 30 min. Recombinant MMP-14 catalytic domain was obtained from Calbiochem, at a concentration of 200 μg/mL. This solution was diluted by half with assay buffer, and 4 μL of the resulting solution was transferred to each probe solution. Aliquots of each solution (25 μL) were removed at time points of 0, 2.5, 5, 7.5, 10, 20, 40, 80, and 120 min, and rapidly transferred to an HPLC vial insert containing 50 μL of ice-cold quenching reagent (0.1 M EDTA/2 Na^+ in 1% HOAc, pH 3). The quenched samples were then analyzed by RP-HPLC (Jupiter C12 analytical column (Phenomenex), flow rate 1.1 mL/min 0–5 min, 95% water, 5% acetonitrile, 0.1% TFA; 5–35 min, ramp to 50% water, 50% acetonitrile, 0.1% TFA; then, re-equilibration at 95% water).

Probe half-lives and initial cleavage velocities were extracted from the analytical HPLC data, assuming enzyme saturation and first-order probe cleavage kinetics early in the reaction time course. For the 4e, 4eg, and 4egg probes, linear curves were constructed by plotting the natural log of the intact probe peak area against time. For these probes, saturation conditions were assumed to persist until the 20 min time point. The 8e probe data was analyzed in a similar fashion, and included the 40 min time point in the analysis. Calculations were conducted twice for each probe, using HPLC data obtained at either 220 or 260 nm. The slope of each linear equation was taken as a measure of initial cleavage velocity. Probe half-lives were estimated by dividing the measured probe peak areas at $t = 0$ in half, and substituting the resulting values into each regression equation to solve for t.

4.4. ^{99m}Tc radiochemistry

Radiolabeling of the probes with ^{99m}Tc was carried out using an IsoLink tricarbonyl labeling kit (Covidien Inc., MO), via a modified procedure. Briefly, a solution of $[^{99m}TcO_4]^-$ (1 mL) from a commercial generator (20–100 mCi) was added to a sealed vial containing sodium boranocarbonate (4.5 mg), sodium tetraborate dodecahydrate (2.85 mg), sodium tartrate dehydrate (8.5 mg) and sodium carbonate (7.15 mg). The reaction mixture was warmed in a boiling water bath for 20 min to form an intermediate complex of $[^{99m}Tc(CO)_3(OH_2)_3]^+$. The pH was adjusted to 6–6.5 using 1 M HCl. This stock solution (200 μL) was then reacted with the unlabeled probe (25–50 nmol dissolved in 100 μL of methanol) at 75 °C for 15–30 min. The final ^{99m}Tc- labeled probe was isolated by HPLC at high purity (90% radiological yield).

4.5. In vitro cell studies

Human epithelial breast cancer (MDA-MB-231) cells transfected with MMP-14 cDNA to overexpress MMP-14 were cultured and characterized using published methods.[41] They were distributed into six-well plates (100,000 cells per well). The plates were incubated overnight. The MMP-14 inhibitor buffer was prepared by dissolving EDTA-disodium salt and GM 1469 metalloprotease inhibitor in DMEM media, to concentrations of 1.0 mM and 0.2 mM, respectively. Aliquots of inhibitor solution (100 μL) were added to three of the wells; the remaining wells received 100 μL aliquots of blank DMEM media. All wells were then incubated for 20 min. Six wells (three treated with inhibitor, three untreated) were spiked with 33 μL (16.5 μL) of ^{99m}Tc radiolabeled probe in PBS. The remaining three wells received 33 μL (16.5 μL) of free ^{99m}Tc tricarbonyl complex in PBS as a negative control. Following 20 min. of incubation time, the medium was removed from each plate, and adhesive cells were washed with an additional 1 mL of DMEM. All media washes were collected in separate microcentrifuge tubes. The adhesive cells were then collected by washing each plate three times with trypsin solution (1 mL per plate; 2 × 2 min, 1 × 30 min), and once with methanol (1 mL per plate; 2 min). Each wash was again collected in a separate microcentrifuge tube. The ^{99m}Tc content of every collected fraction was then quantified by gamma counter.

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Appendix B

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CD4$^+$ T cells Regulate Pulmonary Metastasis of Mammary Carcinomas by Enhancing Pro-tumor Properties of Macrophages

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ABSTRACT

During breast cancer development, increased presence of leukocytes in neoplastic stroma parallels disease progression; however, functional significance of leukocytes in regulating pro-tumor versus anti-tumor immunity in the breast remains poorly understood. Utilizing the MMTV-PyMT model of mammary carcinogenesis, we demonstrate that IL-4-expressing $CD4^+$ T lymphocytes indirectly promote invasion and subsequent metastasis of mammary adenocarcinomas by directly regulating the phenotype and effector function of tumor-associated $CD11b^+Gr1^-F4/80^+$ macrophages that in turn enhance metastasis through activation of epidermal growth factor receptor signaling in malignant mammary epithelial cells. Together, these data indicate that anti-tumor acquired immune programs can be usurped in pro-tumor microenvironments and instead promote malignancy by engaging cellular components of the innate immune system functionally involved in regulating epithelial cell behavior.

Significance: DeNardo and colleagues demonstrate a tumor-promoting role for T_H2-$CD4^+$ T lymphocytes that elicit pro-tumor, as opposed to cytotoxic bioactivities of tumor-associated macrophages and enhancement of pro-metastatic epidermal growth factor receptor signaling programs in malignant mammary epithelial cells. This work reveals a novel pro-tumor regulatory program involving components of the acquired and cellular immune systems that effectively collaborate to promote pulmonary metastasis of mammary adenocarcinomas, and identifies new cellular targets, namely $CD4^+$ T effector cells and IL-4 for anti-cancer therapy.

INTRODUCTION

Clinical and experimental studies have established that chronic infiltration of neoplastic tissue by leukocytes, i.e., chronic inflammation, promotes development and/or progression of various epithelial tumors (de Visser et al., 2006; Mantovani et al., 2008); however, the organ-specific cellular and molecular programs that favor pro-tumor, as opposed to anti-tumor immunity are incompletely understood. While some subsets of leukocytes certainly exhibit anti-tumor activity, including cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells (Dunn et al., 2006), other leukocytes, most notably mast cells, B cells, dendritic cells, granulocytes, and macrophages exhibit more bipolar roles, by virtue of their capacity to either hinder or potentiate tumor progression (de Visser et al., 2005; Mantovani et al., 2008).

Breast cancer development is characterized by significant increases in the presence of both innate and adaptive immune cells, with B and T cells, and macrophages representing the most abundant leukocytes present in neoplastic stroma (DeNardo and Coussens, 2007). Retrospective clinical studies in human breast cancer have revealed that high immunoglobulin (Ig) levels in tumor stoma (and serum), and increased presence of extra follicular B cells, T regulatory (T_{reg}) cells, high ratios of CD4/CD8 or T_H2/T_H1 T lymphocytes in primary tumors or in draining lymph nodes (LNs) correlates with tumor grade, stage and overall patient survival (Bates et al., 2006; Coronella-Wood and Hersh, 2003; Kohrt et al., 2005); thus, some facets of adaptive immunity may indeed be involved in fostering cancer development in the breast.

On the other hand, experimental studies have demonstrated that macrophages in primary mammary adenocarcinomas regulate late-stage carcinogenesis by virtue of their pro-angiogenic properties (Lin and Pollard, 2007), as well as fostering pulmonary metastasis by providing epidermal growth factor (EGF) to malignant mammary epithelial cells (MECs) and thereby enhancing their invasive (and metastatic) behavior (Pollard, 2004). Based on these seemingly disparate observations, we sought to determine if adaptive immunity also fosters malignancy in the breast by regulating the phenotype or effector functions of tumor-associated macrophages (TAMs) and either activating their pro-tumor properties or alternatively by suppressing their anti-tumor capabilities. To address this, we utilized an aggressive transgenic moue model of murine mammary adenocarcinoma development (MMTV-PyMT mice) (Guy et al., 1992) where late-stage carcinogenesis and pulmonary metastasis are regulated by colony stimulating factor (CSF)-1 and tissue macrophages (Lin et al., 2001). We evaluated MMTV-PyMT mice harboring homozygous null mutations in genes regulating development

of specific lymphocyte subtypes and found that $CD4^+$ T cells potentiate pulmonary metastasis of mammary adenocarcinomas indirectly by enhancing aspects of pro-tumor immunity mediated by TAMs.

Results:

$CD4^+$ T cells regulate pulmonary metastasis of mammary adenocarcinomas

As observed in several types of solid tumors, human breast adenocarcinomas are characterized by infiltration of both innate and adaptive immune cells (**Figure 1A**). Immunohistochemical (IHC) detection of $CD68^+$ myeloid cells (macrophages), $CD4^+$ and $CD8^+$ T cells and $CD20^+$ B cells in human breast cancer reveals an increase in each cell type paralleling cancer development (**Figure 1A**). Given the critical role of adaptive immunity in regulating innate immune cell effector function in chronic inflammatory diseases, and in some mouse models of cancer development (de Visser et al., 2005), we hypothesized that B and/or T lymphocytes might exert a functional role in regulating pro-tumor properties of myeloid cells during mammary carcinogenesis. Since infiltration of $CD4^+$ T cells and $F4/80^+$ macrophages increases progressively during mammary carcinogenesis in MMTV-Polyoma Middle T (*PyMT*) mice (**Figure 1B-C**), similar to human breast cancer development (**Figure 1A**), we addressed this hypothesis by generating *PyMT* mice harboring homozygous null mutations in the *recombinase activating gene-1* (*RAG1*) functionally impairing development of B and T cells, i.e., *PyMT/RAG1*$^{-/-}$, and compared them for characteristics of neoplastic progression to *PyMT* mice lacking B cells, i.e., *PyMT/JH*$^{-/-}$, versus selective subsets of T cells, i.e., *PyMT/CD4*$^{-/-}$, *PyMT/CD8*$^{-/-}$ and *PyMT/CD4*$^{-/-}$*/CD8*$^{-/-}$ mice. Strikingly, we found no gross histopathological or quantitative differences between these cohorts when evaluated for primary tumor latency, tumor burden or tumor angiogenesis as a function of complete or selective lymphocyte-deficiency (**Figure 2A-D, Suppl. Figure 1**). In contrast, selective loss of $CD4^+$ T cells in either *PyMT/RAG1*$^{-/-}$, *PyMT/CD4*$^{-/-}$*/CD8*$^{-/-}$, or *PyMT/CD4*$^{-/-}$*/CD8*$^{+/-}$ mice resulted in significant attenuation of pulmonary metastasis including reduced numbers of metastatic foci, decreased tumor burden in lungs (**Figure 2E-F**), decreased presence of circulating $CD45^-$cytokeratin$^+$ carcinoma cells (**Figure 2G),** and cells expressing *PyMT* mRNA (**Figure 2H**) in peripheral blood (PB). To verify that the attenuated metastatic phenotype of *CD4*-deficient/*PyMT* mice was specifically due to lack of $CD4^+$ T cells, as opposed to a genetic anomaly in homozygous null mice, we depleted 85 day-old *PyMT* mice of $CD4^+$ T cells for 25 days and again found reduced pulmonary metastasis (**Figure 2I**) and presence of circulating carcioma cells in PBd (**Figure 2G**). In contrast, adoptive transfer of naive $CD4^+$ T lymphocytes into *PyMT/CD4*$^{-/-}$*/CD8*$^{-/-}$ mice significantly enhanced pulmonary metastasis (**Figure 2I**), together indicating that $CD4^+$ T lymphocytes mediate metastasis of late-stage malignant mammary epithelial cells in a $CD8^+$ CTL-independent manner.

$CD4^+$ T cells regulate macrophage and immature myeloid cell phenotype and effector bioactivity

Since the attenuated metastatic phenotype of *PyMT/$CD4^{-/-}$* mice mirrored tissue macrophage-deficiency previously reported by Pollard and colleagues (Lin et al., 2001), we addressed the possibility that CD4-deficiency might result in altered myeloid cell presence and/or function in late-stage carcinomas. Using flow cytometry and IHC analysis, we found no change in $CD45^+$ leukocyte infiltration in primary adenocarcinomas of 95- and 110 day-old *PyMT* mice resulting from $CD4^+$ T cell-deficiency (**Figure 3A-B**). In addition, using flow cytometry to evaluate the spectrum of leukocytes infiltrating mammary carcinomas, we found no significant variation in leukocyte composition including $CD11b^+Gr1^-F4/80^+$ macrophages or $CD11b^+Gr1^{Hi}F4/80^-$ immature myeloid cells (IMCs) (**Figure 3C**).

Once in tissues however, the differentiation state, phenotype and effector functions of myeloid cells, including macrophages and IMCs can be directly regulated by their immune microenvironment. The bioactive state of macrophages for example, correlates with classical T_H1 and T_H2 nomenclature and is often referred to as M1 (classical) or M2 (alternative) activation, respectively (Mantovani et al., 2007). Classically activated M1 macrophages are regulated by T_H1 cytokines like IFNγ, tumor necrosis factor alpha (TNFα) and granulocyte-monocyte-colony stimulating factor (GM-CSF) that, in part, enhance macrophage cytotoxic activity. In contrast, tissue macrophages exposed to T_H2 cytokines common to tumors, including interleukin (IL)-4, IL-13 or IL-10 manifest an alternative (M2) phenotype that can be potentiated by immune complexes, IL-1, IL-21, transforming growth factor beta (TGFβ), and glucocorticoids. Alternatively activated/M2 macrophages are commonly found associated with solid tumors and are thought to possess immunosuppressive, pro-angiogenic and pro-tissue remodeling bioactivities, as well as expressing high levels of EGF (Leek et al., 2000; Mantovani et al., 2007).

Thus, we evaluated differentiation/maturation and activation status of TAMs (**Figure 3D-L**) and IMCs from carcinomas of 95 day-old CD4-proficient versus CD4-deficient/*PyMT* mice (**Figure 3M-Q**). Expression analysis of lineage differentiation markers, including CD45, F4/80, CD11b and Gr1, in TAMs reveled no significant alteration based on $CD4^+$ T cell presence (**Suppl. Figure 2A-B**). Though similar percentages of $CD45^+CD11b^+Gr1^-F4/80^+$ TAMs infiltrated adenocarcinomas in both cohorts (**Figure 3C**), TAMs of CD4-deficient and RAG1-deficient/*PyMT* mice expressed significantly elevated levels of type 1 cytokines (e.g., TNFα, IL-6, IL-12p40 and IL-1β) and *Nos2* mRNA, indicative of a prevalent M1 TAM phenotype (**Figure 3D-G, Suppl. Figure 2C**) as compared to

TAMs from CD4-proficient/*PyMT* mice (**Figure 3I**). Conversely, expression of factors indicative of alternatively activated (M2) TAMs, including *arginase-1* (*Arg-1*) and *Tgfβ* were significantly reduced in TAMs isolated from mammary tumors of *PyMT/CD4*$^{-/-}$ mice as compared to CD4-proficient littermates (**Figure 3J-K**). Expression levels of IL-10 and *Vegf-a* were similar in TAMs from both cohorts (**Figure 3H and L, Suppl. Figure 2C**). Moreover, cytokine expression of TAMs isolated from *PyMT* mice where CD4$^+$ T cells had been depleted via neutralizing antibodies evidenced similar profiles as observed in TAMs of *PyMT/CD4*$^{-/-}$ mice (**Suppl. Figure 2D-E)**

Yang and colleagues recently reported that CD11b$^+$Gr1^{Hi} IMCs are recruited into mammary carcinomas and regulate pulmonary metastasis in *PyMT* mice via activation of TGFβ-regulated signaling pathways (Yang et al., 2008). In order to determine if CD4$^+$ T cells were also regulating the bioactivity of IMCs, we analyzed their cytokine profile in mammary carcinomas of CD4-proficient versus deficient *PyMT* mice (**Figure 3M-Q, Supp. Figure 2F**) and found significantly elevated expression of factors indicative of an M1 activation state (e.g., TNFα and *Nos2*) with parallel reduction in M2-type factors (e.g., *Arg-1* and *Tgfβ*). Thus, CD4$^+$ T lymphocytes significantly regulate cytokine and mediator expression in both IMCs and TAMs in mammary adenocarcinomas.

TAM phenotype in mammary carcinomas is T_{reg}*-independent*

In vitro, both CD4$^+$ T effector and T_{reg} cells have the capacity to modulate macrophage cytokine expression (Tiemessen et al., 2007). In order to determine which of these populations were regulating TAM bioactivity *in vivo*, we immune-depleted CD25$^+$ T_{regs} by treating cohorts of 80 day-old *PyMT* mice with anti-CD25 IgG (PC61), versus isotype control Ig, for 20 days and found no differences in expression of M1-type cytokines (e.g., TNFα, IL-6, IL-12p40, *IL-12p35*, *Nos2)* or M2-induced genes *Arg1, IL-10* or *Tgfβ* **(Suppl. Figure 3A-C)**. Thus, CD4$^+$ T effector lymphocytes, but not CD25^{Hi} T_{reg} cells, significantly regulate TAM phenotype and bioeffector function.

CD4$^+$ *T lymphocytes in mammary adenocarcinomas express* T_H2 *cytokines.*

To determine if CD4$^+$ T cells regulated TAM phenotype by a T_H2 cytokine-mediated pathway, we evaluated mRNA expression of CD4$^+$ T cells isolated from LNs and mammary carcinomas of 95 day-old *PyMT* mice (**Suppl. Figure 4A-B)** for transcription factors and effector molecules indicative of T_{reg}, T_H1, T_H2 or T_H17-type responses. CD4$^+$ T lymphocytes isolated from draining LNs (LNs) and mammary carcinomas of *PyMT* mice exhibited elevated expression of *GATA3* (T_H2) and *T-bet* (T_H1)

mRNA, but not *FOXP3* (T_{reg}), when compared to LNs of wild type littermates (**Figure 4A-C**) indicating that both T_H1 and T_H2 effector lineages were expanded in LNs and in tumors. In order to assess the functional consequences of these, we assessed the cytokine expression profile of $CD4^+$ cells and found significant induction in T_H2 cytokines including *IL-4*, *IL-13*, and *IL-10* and to a lesser extent the T_H1 cytokine *IFNγ*, and by contrast, *IL-17a* was not significantly expressed (**Figure 4E-H**). These results were further confirmed by *ex vivo* activation of $CD4^+$ T lymphocytes (isolated from spleen, draining LNs and tumors of *PyMT* mice) with anti-CD3/CD28 Igs. Analysis of expression of IL-4, IFNγ and IL-17 by ELISA, and IFNγ and IL-4 by intracellular flow cytometry, and found that activated $CD4^+$ T cells expressed higher levels of IL-4 as compared to IFNγ or IL-17 (**Figure 4I**), and that IL-4-expressing $CD4^+$ T cells represented a larger fraction of the total $CD4^+$ T cells present in mammary tumors *in vivo* (**Suppl. Figure 5A**).

To determine if IL-4 produced by $CD4^+$ T cells was involved in differentially regulating macrophage effector functions, we assessed M1/M2 cytokine profile of TAMs isolated from CD4-deficient and CD4-proficient/*PyMT* mice using an *ex-vivo* assay. We found that brief exposure of primary TAMs (isolated from *PyMT/CD4*$^{-/-}$ or *PyMT/RAG1*$^{-/-}$ adenocarcinomas) to exogenous IL-4 resulted in significantly reduced M1-type cytokine expression, simultaneous with enhanced expression of M2-type factors and mirroring cytokine expression of TAMs from CD4-proficient/*PyMT* mice (**Figure 3D-L, Suppl. Figure 2C-F**), thus indicating a dual role for IL-4 and perhaps $CD4^+$ T cells in regulating macrophage polarity.

We next determined if tumor-associated $CD4^+$ T lymphocytes by virtue of their expression of IL-4 directly repressed TAM M1 phenotype *ex vivo*. Whereas IFNγ/LPS treatment of TAMs led to increased TNFα and IL-12 expression (indicative of M1 activation), this effect was repressed in the presence of activated $CD4^+CD25^-$ T effector cells (**Figure 4J-K**), thus indicating that tumor-associated $CD4^+$ T lymphocytes actively repress M1 TAM effector function, while simultaneously fostering a pro-tumor alternative/M2 TAM phenotype via expression of cytokines like IL-4.

$CD4^+$ T cells regulate macrophage-induced MEC invasive behavior

To reveal if $CD4^+$ T cell activation of TAMs translated into enhanced invasive behavior of mammary epithelial cells (MECs), a requirement for metastasis *in vivo*, we utilized an *ex vivo* three-dimensional (3D) organotypic co-culture model with primary murine cells. Primary MECs were isolated from either 76 day-old *PyMT* mice (pMECs) or 12-week old virgin negative littermates (nMECs), placed in

3D overlay culture and allowed to form stable non-invasive organoids (**Figure 5A**) as previously described (Debnath et al., 2003). Following formation of stable organoids (2-3 weeks), $CD45^+CD11b^+Gr1^-F4/80^+$ TAMs isolated from mammary carcinomas of 95 day-old *PyMT* mice (**Suppl. Figure 2A-B**) were added, resulting in elaboration of an invasive MEC phenotype in a significant percentage of organoids (**Figure 5A**). When invasive pMEC organoids formed, TAMs were typically localized at the "invasive fronts" of invading structures (**Figure 5A, panels *c-f***). In addition, when TAMs were co-cultured with pMEC organoids in the presence of T_H2-type cytokines (IL-4 or IL-13), organoid disruption and formation of invasive structures was significantly enhanced (**Figure 5B**) in a TAM and IL-4 dose-dependent manner (**Suppl. Figure 6A-B**). In contrast, when IL-4 or IL-13 cytokines were added to organoids alone (without TAMs), no significant change in organoid stability or invasive behavior was observed (**Figure 5B**) indicating that cytokine stimulation of pMEC invasion was mediated by TAMs. Analogous results were found utilizing IL-4-activated TAMs to induce pMEC invasion in standard Boyden Chamber migration assays (**Suppl. Figure 6C**). In contrast, when TAMs were co-cultured with pMEC organoids in the presence of M1-type cytokines (IFNγ or LPS) or the immunosuppressive cytokine IL-10, the invasive pMEC phenotype was significantly inhibited and instead additional stability to organoids was observed (**Figure 5B**). Moreover, to evaluate if tumor-associated $CD4^+$ T cells were involved or perhaps directly regulating TAM-induced MEC invasion, TAMs and pMEC organoids were "tri-cultured" with $CD4^+$ T cells isolated from mammary carcinomas of 95 day-old *PyMT* mice (**Suppl. Figure 4A**) resulting in a significant enhancement of pMEC invasive organoids in an IL-4 dependent manner (**Figure 5C**). Taken together with data from the *in vivo* analysis of TAM phenotype, these data indicate that tumor-associated TAMs are alternatively (M2) activated by IL-4-expressing $CD4^+$ T cells, that together induce invasive behavior of MECs, a bioactivity that is not supported by TAMs isolated from $CD4^+$ T cell-deficient adenocarcinomas, or when TAMs are 'classically' activated by factors like IFNγ or LPS, or engaged in immunosuppressive programs regulated by IL-10.

IL-4 regulates TAM phenotype and pulmonary metastasis of mammary adenocarcinomas

Since the capacity of tumor-associated $CD4^+$ T lymphocytes to regulate TAM phenotype and pMEC invasion was dependent on T_H2-type cytokines, we hypothesized that neutralization of IL-4 or its receptors *in vivo* would mirror the phenotype of CD4-deficient/*PyMT* mice and limit pulmonary metastasis. To address this, we generated *PyMT* mice either harboring a homozygous inactivating

mutation in the *IL-4 receptor alpha* (*IL4Rα*) gene (FVB/n, N6), or treated *PyMT* mice with a neutralizing antibody to IL-4. Similar to $CD4^+$ T cell-deficient/*PyMT* mice, both cohorts of *PyMT/IL4Rα*$^{-/-}$ and IL-4-neutralized/*PyMT* mice exhibited no significant change in primary tumor latency or burden as compared to controls (**Figure 6A-B**). However, loss of either IL-4 activity or expression of *IL4Rα* resulted in significantly reduced numbers of metastatic foci in lungs and overall attenuation of total pulmonary metastasis (**Figure 6C**). Moreover, cytokine analysis of TAMs isolated from both *PyMT/IL4Rα*$^{-/-}$ and IL-4-depleted/*PyMT* mice revealed increased expression of M1-type factors (IL-6, *Nos2*, *IL-12p35*) and reduced expression of M2-type genes (*Arg1* and *Tgfβ*) as compared to TAMs from control mice (**Figure 6D-E)** and thus phenocopied the characteristics of TAMs isolated from $CD4^+$ T cell-deficient/*PyMT* mice (**Figure 3)**.

IL-4 signaling induces macrophage EGF mRNA expression and EGFR-dependent invasion and metastasis

Since elaboration of the invasion MEC phenotype certainly involved activation of intracellular MEC signal transduction programs, we next sought to identify the soluble mediators released by TAMs following their activation by IL-4 and $CD4^+$ T cells. Thus, we assessed expression of several growth factors associated with epithelial cell invasion and found that TAMs isolated from CD4-proficient/*PyMT* mice exhibited elevated levels of *EGF* and *Tgfβ* mRNA expression, as compared to TAMs from $CD4^+$ T cell-deficient/*PyMT* mice (**Figure 3K, Figure 7A** and data not shown). In addition, TAMs represent the most abundant cellular source of *EGF* mRNA in mammary carcinomas (**Supp. Figure 6E**). To determine if enhanced *EGF* mRNA expression by TAMs was directly due to IL-4 exposure, we evaluated *EGF* mRNA expression of TAMs following brief exposure to IL-4, CSF-1, IL-4 plus CSF-1, as compared to pMEC conditioned medium alone, and found that *EGF* mRNA expression was significantly enhanced by IL-4, but only in the presence of CSF-1 or pMEC conditioned medium (**Figure 7A, Suppl. Figure 6D**).

To establish if activation of EGF receptor (EGFR)-mediated signaling was necessary for TAM-induced pMEC invasion, we evaluated effects of EGFR blockade using the 3D co-culture assay and found that IL-4-regulated TAM-dependent pMEC invasion was significantly diminished in the presence of EGFR tyrosine kinase inhibitors (**Figure 7B**). To determine if this translated to a diminishment in metastasis *in vivo,* late-stage (day 110) *PyMT* mice were treated with PD153035 (25 mg/kg) for 5 hours and the presence of circulating malignant cells quantitatively determined. This

brief treatment resulted in a significant decrease in the number of circulating carcinoma cells present in PB (**Figure 7C**), similar to observations by Wyckoff and colleagues (Wyckoff et al., 2007). Taken together, these data indicate that in response to $CD4^+$ T cell-derived IL-4, M2 effector bioactivity is enhanced in TAMs (and IMCs) that in turn activates invasive and metastatic potential of MECs in mammary adenocarcinomas through their production of pro-invasive/metastatic factors such as EGF (**Figure 7D**).

DISCUSSION

We revealed a provocative and functional role for $CD4^+$ T effector cells as potentiators of PB dissemination and pulmonary metastasis of mammary adenocarcinomas through their ability to regulate pro-tumor properties of TAMs. Specifically, T_H2-polarized $CD4^+$ T lymphocytes regulate M1 and M2-type TAM bioactivity by their expression of IL-4. M2-TAMs in turn promote invasive behavior of malignant MECs by high level production of EGF that subsequently activates MEC EGFR signaling programs, an activity essential for entry into PB, dissemination and outgrowth in the lung. These findings indicate that when $CD4^+$ T lymphocytes are present in a T_H2-type tumor microenvironment, they can promote metastasis by regulating the pro-tumor properties of TAMs, as opposed to limiting or eradicating malignant cells by engaging cytotoxic mechanisms. This realization provides rational for development of anti-cancer therapeutics that neutralize the pro-tumor properties of both adaptive and innate immune cells in the tumor microenvironment, that when delivered in combination with cytotoxic drugs that bolster anti-tumor immunity, may thereby extend survival of breast cancer patients with advanced disease.

Effector function of $CD4^+$ T cells in solid tumors is context-dependent

While our studies have revealed that $CD4^+$ T cells potentiate dissemination and metastasis of mammary adenocarcinomas, it is clear that $CD4^+$ T lymphocytes in other contexts exhibit other bioactivities. Schreiber and colleagues demonstrated that $CD4^+$ T cell-deficiency in methylcholanthrene (MCA)-initiated sarcomas enhanced tumor development (Koebel et al., 2007). By contrast, following two-stage skin carcinogenesis (Dimethylbenzanthracene plus Tetradecanoylphorbol-acetate), $CD4^+$ T cell-deficiency was associated with diminished tumor development (Girardi et al., 2004). Thus, tumor etiology, in combination with the tumor microenvironment together regulates $CD4^+$ T cell phenotype, and in part determines whether a pro-tumor, as opposed to an anti-tumor immune program is favored. In agreement with the tissue context-dependent nature of $CD4^+$ T cells, in a mouse model of skin and cervical carcinoma development where oncogenes from human papilloma virus type 16 are expressed behind the keratin 14 promoter/enhancer, skin carcinoma formation is modestly attenuated by $CD4^+$ T cell-deficiency, whereas cervical carcinoma development is significantly enhanced (Daniel et al., 2005; Daniel et al., 2003), again demonstrating that immune responses accompanying tumor development are organ-

dependent as opposed to oncogene-dependent, and based on the neoplastic and immune microenvironment, can engage either pro- or anti-tumor immune regulatory programs.

$CD4^+$ T lymphocytes have been traditionally classified as either tumor suppressive, such as T_H1 effector cells that repress tumor growth by secretion of IFNγ (amongst other soluble mediators) and support of CTL function, or alternatively as tumor-promoting cells, including T_{regs}, that foster tumor expansion by suppressing $CD8^+$ CTLs and NK cells (Trzonkowski et al., 2006). The interplay between T_H1 and T_{regs} in regulating tumor immunity is likely critical for the etiology of some malignancies, such as sarcomas (Dunn et al., 2006; Koebel et al., 2007) or lung adenocarcinomas (Woo et al., 2002). In addition to these, a new class of $CD4^+$ T cells expressing IL-17, i.e. T_H17 cells, have been identified that may also regulate chronic inflammation and promote tumor development when activated in the presence of TGFβ and IL-6, or IL-23 (Dong, 2008). In our studies, we found that pulmonary metastasis and M2-bioactivity of TAMs was potentiated by $CD4^+$ T effector cells that express high levels of IL-4, IL-13 and IL-10, as compared to expression of IFNγ or IL-17 (**Figure 3-4**), whereas TAM bioactivities were unaffected by immune-depletion of $CD25^+$ T_{reg} cells. Moreover, $CD4^+$ T cells exerted these affects independently of the presence or absence of $CD8^+$ T cells indicating their pro-tumor functionality does not involve suppression of CTL activity.

In addition to indirectly potentiating cancer development by regulating pro-tumor properties of myeloid cells, research from several laboratories has revealed that IL-4 and IL-13 regulate tumor growth through activation of IL-4/13 receptors on epithelial cells. In some human breast carcinoma cell lines, particularly those that express the estrogen receptor α, IL-4 and IL-13 inhibit basal and estrogen-induced cell proliferation *in vitro* and in xenograph models *in vivo* (Gingras et al., 2000; Gooch et al., 2002; Nagai and Toi, 2000). However, in other breast carcinoma cell lines, IL-4 regulates tumor cell survival by conferring resistance to apoptosis (*in vitro*) that translates to chemo-resistance in xenographs (Todaro et al., 2008). Palucka and colleagues reported that $CD4^+$ T cells directly enhance early tumor development by their production of IL-13 (Aspord et al., 2007). In contrast to these, we found no change in the latency or development of primary mammary adenocarcinomas due to either $CD4^+$ T cell or IL4Rα-deficiency, indicating that in this model system, $CD4^+$ T cells and IL-4 likely do not provide a survival or proliferative advantage directly to neoplastic cells. Moreover, using the *ex vivo* cell-based assay, we also found no change in MEC proliferation, acinar morphology or organoid stability when co-cultured with $CD4^+$ T cells alone, or when MECs were given IL-4 or IL-13. Instead, when cultured together with MECs in the presence of TAMs, either

tumor-derived $CD4^+$ T cells, IL-4 or IL-13 induced significant changes in organoid morphology consistent with invasive growth (**Figure 5**). Taken together these data indicate that the effects of CD4 T cell-derived T_H2 cytokines on tumor development and progression is likely regulated by the organ microenvironment or IL-4/13 receptor status on cell in the tumor microenvironment.

Clinical evaluation of human breast cancers has revealed that presence of $CD4^+$ T_H2 and T_{reg} cells increase during cancer development. High percentages of $CD4^+$ T cells positively correlate with tumor stage, including metastatic spread to sentinel LNs and increased primary tumor size (Kohrt et al., 2005). Perhaps more significant, the ratio of $CD4^+$ to $CD8^+$ T cells or T_H2 to T_H1 cells in primary tumors, where $CD4^+$ or T_H2 cells are more frequent than $CD8^+$ or T_H1 cells, correlates with LN metastasis and reduced overall patient survival (Kohrt et al., 2005). More recently, unsupervised expression profiling from breast cancer-associated stroma revealed a gene signature predictive of good prognostic outcome (>98%, 5 year survival) that was functionally enriched for elements of a T_H1-type immune response, including genes suggestive of CTL and NK cell activity (Finak et al., 2008). Conversely, high levels of $FOXP3^+$ T_{reg} cells predict diminished relapse-free and overall survival (Bates et al., 2006). The interpretation based upon these clinical studies is that the type of $CD4^+$ effector T cell response elicited in an emergent breast cancer may in part determine malignant and metastatic potential. Our data provide some clarity to these profiles wherein we report that T_H2-$CD4^+$ T cells promote metastasis, not by altering CTL responses, but instead by enhancing the pro-tumor bioactivities of myeloid cells, and enhancing intracellular signaling cascades (EGF) required for dissemination and metastasis.

Macrophage–mediated pro- versus anti-tumor immunity

Macrophages promote metastasis in several contexts, i.e., by supporting tumor-associated angiogenesis, inducing local immunosuppression, or by promoting malignant cell invasion and entry into circulation (Condeelis and Pollard, 2006); however, the molecular mechanisms regulating each of these “hallmark” pro-tumor TAM properties have yet to be elucidated. Macrophages are implicated in tumor angiogenesis (a prerequisite for metastasis) by virtue of their capacity to express pro-angiogenic factors including VEGF and matrix metalloproteinase (MMP)-9 (Giraudo et al., 2004), and by clinical data in human breast cancers demonstrating their presence correlates with increased micro-vessel density (Uzzan et al., 2004). Accordingly, tissue macrophage-deficiency in *PyMT* mice leads to reduced angiogenesis, delayed onset of late-stage carcinomas and greatly diminished pulmonary

metastasis (Lin and Pollard, 2007). By comparison, loss of $CD4^+$ T lymphocytes, similar to loss of tissue TAMs, results in reduced presence of circulating carcinoma cells and diminished pulmonary metastasis, but did not impact microvessel density, character of angiogenic vasculature or expression of *Vegf-a* or *MMP-9* by TAMs or IMCs. These distinctions reflect the fact that $CD4^+$ T cell-derived factors, including IL-4, regulate only some aspects of TAM bioactivity, in particular invasive and metastatic properties of MECs that are EGF-dependent; thus, pro-angiogenic TAMs are likely regulated by other factors such as hypoxia.

TAMs exhibit immunosuppressive activity via their expression of arginase, IL-10 and TGFβ (Mantovani et al., 2007). Our data demonstrate that $CD4^+$ T cells and IL-4 induce some but not all of their immunosuppressive properties, specifically expression of arginase-1, TGFβ and IL-12, but not IL-10, MHCII or CD86 (**Figure 3** and data not shown). Mantovani and colleagues reported that some aspects of TAM-mediated immunosuppression are regulated by intracellular NF-κB signaling (Saccani et al., 2006). Moreover, Balkwill and colleagues revealed that ovarian cancer-associated TAMs, due to IL-1R and MyD88, maintain an immunosuppressive M2 phenotype dependent on IKKβ (Hagemann et al., 2008). *In vivo*, IKKβ$^{-/-}$deficient TAMs instead exhibit tumor cell cytotoxicity and switch to a classically activated M1 phenotype (e.g., IL-12^{high}, major histocompatibility complex II^{high}, IL-10^{low}, arginase-1^{low}) that promotes regression of advanced ovarian carcinomas by induction of TAM tumoricidal activity and activation of IL-12-dependent NK cell recruitment (Hagemann et al., 2008). The implication from these experimental findings imply that reprogramming TAM phenotype and/or altering the immune microenvironment to foster anti-tumor activity would diminish tumorgenicity and could improve clinical outcome.

Mechanisms of TAMs induced epithelial cell invasion and metastasis

Reciprocal interactions between TAMs and MECs together regulate mammary carcinogenesis through activation of a paracrine feed-forward loop involving TAM-expressed *EGF* and epithelial-expressed CSF-1 (Wyckoff et al., 2004). This paracrine loop is critical for branching morphogenesis (Gouon-Evans et al., 2000), as well as for breast carcinoma cells exhibiting "high-velocity" polarized movement (chemotaxis) along collagen fibers towards blood vessels directed by perivascular macrophages (Wyckoff et al., 2007). We propose that these heterotypic interactions are further regulated by factors derived from $CD4^+$ T lymphocytes including IL-4, IL-13 and possibly IFNγ. Herein, we demonstrated that activation of TAMs by IL-4, in combination with factors derived from

malignant MECs such as CSF1, regulate high-level expression of *EGF*, that in turn stimulates EGFR-induced MEC invasive behavior *in vitro* and MEC entry into PB and pulmonary metastasis *in vivo*. As such, a T_H2-rich microenvironment likely collaborates with existing genetic mutations in neoplastic cells, and thereby foster development of highly invasive tumors *in vivo.*

In addition to EGF, production of TGFβ by M2 TAMs, mesenchymal support cells and IMCs also enhances invasive and metastatic programming of malignant cells (Yang et al., 2008). Profiling of human breast carcinomas has revealed that a TGFβ-responsive gene signature predicts lung metastasis (Padua et al., 2008). Similarly, we found that the absence of $CD4^+$ T cells also resulted in decreased *Tgfβ* expression in TAMs (and IMCs) in mammary adenocarcinomas. The prediction based on these data is that the type of $CD4^+$ effector lymphocyte response elicited by the neoplastic microenvironment functionally modulates critical stromal derived factors, such as EGF and TGFβ, that collaborate with tumor cell-intrinsic programs to regulate invasive and metastatic potential.

Summary

Taken together with clinical and experimental studies, our data indicate that $CD4^+$ T effector lymphocytes potentiate mammary adenocarcinoma metastasis by modulating the pro-tumor properties of TAMs that in turn enhance the invasive potential of malignant mammary epithelial cells. Since late-stage immune-depletion of $CD4^+$ T cells or IL-4 resulted in a significant diminution in circulating malignant carcinoma cells and reduced outgrowth of pulmonary metastases, these provocative findings indicate that anti-cancer therapeutic strategies targeting the effector bioactivity of T cells may hold promise for treating late-stage disease. While ongoing genetic alterations clearly play a role in regulating the malignant behavior of a neoplastic cell, our study in combination with others revealing dominant roles played by the tumor microenvironment in regulating malignancy, support the long-standing hypothesis (Bissell et al., 1982) that the *host* response and microenvironment in which a neoplastic cell evolves is as critical to its evolution as the genetic changes occurring within its nucleus.

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METHODS

Animal husbandry

Mice carrying the *PyMT* gene under the control of the MMTV promoter in the FVB/n background, were obtained from Dr. Zena Werb (UCSF, San Francisco, CA) and have been previously described (Guy et al., 1992). Generation and characterization of FVB/n mice homozygous null (−/−) for *RAG-1*, *CD4* and *CD8* have been described previously (de Visser et al., 2005). Homozygous null JH and IL4Rα mice were obtained from Jackson laboratories. To generate *PyMT* mice on the *RAG-1*$^{-/-}$, *JH*$^{-/-}$, *CD4*$^{-/-}$, *CD8*$^{-/-}$ *and IL4Rα*$^{-/-}$ backgrounds, *RAG-1*$^{+/-}$, *JH*$^{+/-}$, *CD4*$^{+/-}$, *CD8*$^{+/-}$ and *IL4Rα*$^{+/-}$ mice were backcrossed into the FVB/n strain to N15, N5, N14, N7, and N6 respectively, and then intercrossed with *PyMT* mice to generate breeding colonies of -/- and +/- *PyMT/RAG-1*, *PyMT/JH*, *PyMT/CD4*, *PyMT/CD8*, *PyMT/CD4/CD8* and PyMT/*IL4Rα* mice. Immune depleted mice were injected every 5 days *i.p.* with either anti-CD4 (400 μg, GK1.5), ant-CD25 (400 μg, PC61), anti-IL-4 (1.0 mg, 11B11) or control rat Ig. All mice were maintained within the UCSF Laboratory for Animal Care barrier facility according to IACUC procedures.

Primary and organoid cell culture

Primary nMEC and pMEC pools were established by organoid centrifugation as previously described (Pullan, 1996). Briefly, mammary tissue biopsies were harvested from 76 day-old PyMT female or 12 week old virgin negative littermates and digested with Collagenase A 2.0 mg/ml (Roche) and DNAse 2.0 mM/ml (Roche) for 2 hours. Organoids were then isolated by differential centrifugation and grown in culture conditions as previously described (Pullan, 1996). Primary nMECs were used within 2 passages and primary pMEC cells were used within 10 passages. 3D organotypic cultures were established as previously described (Debnath et al., 2003; Lee et al., 2007). Cultrex basement membrane extract (BME; R&D Systems) was utilized to limit endotoxin levels. Co-cultures with primary leukocytes were established only after stable organoid structures had formed (approximately 3 weeks for nMEC, 2 weeks for pMEC). Leukocytes were overlaid in medium containing 0.5 % BME. Formation of invasive acini was assessed every 12 hours for 3 days. The cytokines IL-4 (20 ng/ml), IL-13 (20 ng/ml), IL-10 (10 ng/ml), IFNγ (5.0 ng/ml) (Peprotech) or LPS (1.0 mM/ml) were added to co-cultures 12 hours after leukocytes overlay. Inhibitors PD153035 (0.1 mM, Calbiochem) or BIBX1382 (10 nM, Calbiochem) were added 1.0-hour prior to the addition of leukocytes. All

experiments were repeated 2-3 times with separate pMEC pools and individual experiments were run in at least in triplicate.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism and/or InStat Software. Specific tests used were Student *t-test*, Mann-Whitney (unpaired, nonparametric, two-tailed), unpaired t-test Welch corrected, generalized Wilcoxon test and Log rank analysis. p values < 0.05 were considered statistically significant

Additional methods are included in supplemental data.

FIGURE LEGENDS:

Figure 1. Concomitant recruitment of adaptive and innate immune cells in breast cancers

A) The number of $CD68^+$, $CD20^+$, $CD4^+$ and $CD8^+$ cells was analyzed in patient samples of normal/hyperplastic breast tissue (n/h; n=9), ductal carcinoma *in situ* (DCIS; n=6) and invasive ductal carcinomas (IC; n=150) using tissue micro-arrays. Representative 10x and 40x images are shown and the average number of positive cells as depicted reflects the mean number of cells in each disease stage, evaluated by counting all high power fields (20x) per tissue section (1.1 mm)/2 sections/patient. * $p<0.05$ by Mann-Whitney.

B-C) $CD4^+$ and $F4/80^+$ cell presence was evaluated during MMTV-PyMT mammary tumor development and is depicted by representative images in normal mammary tissue (-LM) and tumors from 76- and 110 day-old *PyMT* mice. $CD4^+$ or $F4/80^+$ cells were quantitatively assessed and data reflects the mean number of positive cell evaluated in 10 high power fields (20x) per tumor, n=4 mice per group.

Figure 2. $CD4^+$ T cells promote metastasis but not primary tumor development.

A) Mammary adenocarcinoma incidence in *PyMT/RAG1*$^{+/-}$ and *PyMT/RAG1*$^{-/-}$ mice (n=15 and 18 mice/group, respectively) is depicted as % of tumor-free mice. Mice were considered to be tumor free until a palpable mass (>4.0 mm) persisted for longer then 4 days. No statistical differences between cohorts were observed as evaluated by Wilcoxon test.

B-D) Total tumor burden of *PyMT/RAG1* **(B)**, *PyMT/JH* **(C)**, and *PyMT/CD4/CD8* **(D)** cohorts evaluated at both 95- and 110 days of age, shown as mm^3 (n = >20 mice per cohort). Tumor size was determined by caliper measurement and multiple tumors in one animal were added together. No statistical differences between groups were found as evaluated by Mann-Whitney test.

E) Representative lung tissue sections depicting metastatic tumor burden from 110 day-old *PyMT/RAG1*$^{-/-}$, *PyMT/JH*$^{-/-}$, *PyMT/CD4*$^{-/-}$*/CD8*$^{-/-}$ mice following H&E staining (5x magnification).

F) Quantification of metastatic foci/lung section/mouse from 110 day-old *PyMT/RAG1*, *PyMT/JH*, *PyMT/CD4/CD8, PyMT/CD4, PyMT/CD8* cohorts. Each lung was serially sectioned and 6 sections 100 μm apart were H&E stained and total number of metastatic foci (greater then 5 cells) quantified. Each of the six sections was averaged per mouse (n = >20 mice per cohort).

G) Circulating carcinoma cells were analyzed by flow cytometry and counted as the number of cytokeratin$^+$/CD45$^-$ cells in blood from 110 day-old *PyMT/RAG1*$^{+/-}$(n=10), *PyMT/RAG1*$^{-/-}$ (n=10),

PyMT/CD4$^{+/-}$ (n=20), *PyMT/CD4*$^{-/-}$ (n=15) or 110-day old *PyMT* mice treated with anti-CD4 depleting IgG (n=8) or IgG control (n=6) for 18 days. Data are depicted as the mean number of carcinoma cells/ml of blood.

H) *PyMT* mRNA expression in circulating blood cells. RNA from whole blood cells of 110 day-old *PyMT/RAG1*$^{+/-}$ and */RAG1*$^{-/-}$ mice was evaluated for *PyMT* mRNA gene expression by RT-PCR (25 cycles) (n=8 mice/group). Results from ethidium bromide stained gels are depicted following quantification of pixel density using GelDoc software .

I) Average number of metastatic foci/lung section/mouse from 110 day-old *PyMT/CD4*$^{+/-}$ mice treated with anti-CD4 depletion antibody (GK1.5) versus IgG control (CD4$^{+/-}$ IgG) or *PyMT/CD4*$^{-/-}$*CD8*$^{-/-}$ mice following adoptive transfer of naive CD4^{+} T cells (CD4^{+} rescue). Each lung was serially sectioned and accessed as described above. 20 mice were used for *PyMT/CD4*$^{-/-}$*CD8*$^{-/-}$, 6 mice for CD4^{+} rescue, and 8 mice for CD4$^{+/-}$ IgG or GK1.5 groups.

B-I) SEM is shown and * denotes $p<0.05$ by Mann-Whitney

Figure 3. CD4^{+} T lymphocytes do not regulate leukocyte infiltration but instead regulate bioeffector function of myeloid cell subsets.

A) Immuno-detection of CD45^{+} cells in 95 day-old *PyMT/RAG1*$^{+/-}$ and *PyMT/RAG1*$^{-/-}$ mammary carcinomas. Representative 20x images are shown.

B) Flow cytometric analysis of CD45^{+} cells in tumors from 95- and 110 day-old *PyMT/RAG1* and *PyMT/CD4* mice. Data is depicted as the mean percent of live cells ± SEM, n=4 mice per cohort.

C) Flow cytometric analysis of individual leukocyte populations as a percent of total CD45^{+} cells in mammary carcinomas of *PyMT* mice during progression and in day 95 tumors from *RAG1*- and *CD4*-deficient/*PyMT* mice. Data is depicted as the mean value from 4 mice/cohort ± SEM. No statistical differences were found between groups by Mann-Whitney test.

D-L) Cytokine expression by TAM. Tumor-associated CD45^{+}F4/80^{+}Gr1^{-} macrophages were isolated by dual magnetic and flow sorting of mammary tumors from 95 day-old *PyMT/CD4*$^{+/-}$ and *PyMT/CD4*$^{-/-}$ mice (n=3/cohort). Cytokine expression (TNFα, IL-6, IL-12p40, IL-1β, IL-10) was assessed by ELISA of conditioned medium or by qRT-PCR (*Nos2, Arg1, Tgfβ* and *Vegf-a*) from TAMs (50,000) following 18 hours of culture with or without exogenous recombinant IL-4 (10 ng/ml). Representative assays of mammary carcinomas from 3-4 mice evaluated independently in triplicate and depicted as mean ± SEM.

M-Q) Analysis of tumor-derived IMC phenotype in *PyMT/CD4*$^{-/-}$ mice. Tumor-associated CD45^{+}CD11b^{+}Gr1^{Hi} IMCs were isolated by flow from mammary tumors of 95 day-old *PyMT*$^{+}$*/CD4*$^{+/-}$ and *PyMT/CD4*$^{-/-}$ mice (n=3/cohort). Isolated cells were lysed and RNAs assessed by qRT-PCR as described above. Representative assays from 2 independent cohorts each run at least in triplicate are depicted as mean values ± SEM.

* denotes $p<0.05$ by Mann-Whitney in all panels.

Figure 4. CD4^{+} T lymphocytes are T_H2 cells in primary mammary carcinomas

A-H) Analysis of cytokine expression by tumor-associated CD4^{+} T cells. CD4^{+} T cells were isolated by flow sorting from LNs and tumors of 95 day-old *PyMT* mice and corresponding negative littermates (n=4/cohort). Sorted cells were lysed and RNAs were assessed by qRT-PCR for *GATA3, T-bet, FOXP3, IFNγ, IL-4, IL-13, IL-10 and IL-17a* expression. Data is depicted as the mean fold change from the standardized sample (-LM LN).

I) Cytokine analysis in CD4^{+} T cells *ex vivo*. Tumor-associated CD4^{+} lymphocytes were isolated by flow sorting from mammary tumors of 95 day-old *PyMT/CD4*$^{+/-}$ mice (n=3) and *IL-4*, *IFNγ* and *IL-17* expression assessed by ELISA after 18 hours of culture prior to (veh, white bars) or following TCR activation (black bars). Data is represented as the mean of three replicates.

J-K) CD4^{+} T cells repress TAM M1 phenotype. Tumor-associated CD45^{+}CD3^{+}CD4^{+} T lymphocytes and CD45^{+}F4/80^{+}Gr1^{-} TAMs were isolated by flow sorting from mammary tumors of 95 day-old *PyMT/CD4*$^{+/-}$ mice. TAMs were untreated (V, white bars), or cultured with IFNγ (5 ng/ml) and LPS (50 ng/ml) in the presence of control CD4^{+} T cells (treated with control IgGs, grey bars), or activated CD4^{+} T cells (black bar, Act-CD4). TNFα and IL-12p40 expression in conditioned medium evaluated by ELISA after 18 hours of co-culture. Representative data from 2 independent experiments is depicted.

A-K) SEM is shown and * denotes $p<0.05$ by Mann-Whitney

Figure 5. M2-activated TAMs induce invasive behavior in 3D mammary epithelial organoids.

A) Quantitation of invasive organoids following co-culture of TAMs. Stable wildtype MEC (nMEC) or PyMT-derived MEC (pMEC) organoids were allowed to form over 14-20 days and then co-cultured with TAMs (48 hours). Representative immuno-fluorescent images of nMEC (a, c) and pMEC (b, d) organoids in the presence or absence of TAMs evaluated for of cytokeratin 7 (Green), F4/80 (Red) and

DAPI (Blue) are shown. Representative bright field images of invasive pMEC organoids in co-culture with TAMs are depicted at 20x magnification and 40x inset. TAMs are denoted by red arrows.

B) Quantification of organoid disruption following co-culture of TAM with pMEC spheroids in the presence of IL-4 (20 ng/ml), IL-13 (20 ng/ml), IL-10 (10 ng/ml), IFNγ (5 ng/ml) or LPS (50 ng/ml). .

C) Quantification of pMEC organoid disruption (formed over 14 days) following co-culture (48 hours) with TAMs and/or tumor–associated $CD3^+CD4^+$ T cells. Co-cultures were also exposed to exogenous recombinant mouse IL-4 (10 ng/ml) and/or an anti-mouse IL-4 neutralizing antibody (0.5 mg/ml; clone OP06) added 12 hours prior to leukocytes.

A-C) Invasive organoids were quantified and data graphed as a percentage of the total organoids (>100 replicate). Representative data from 2 independent experiments performed in triplicate are depicted as mean. Quantitative data are represented as mean ± SEM and * denotes $p<0.05$ by Mann-Whitney.

D-E) Tumor-associated $CD45^+F4/80^+Gr1^-$ macrophages were isolated by flow sorting of mammary carcinomas from **(D)** *PyMT/IL-4Rα* or **(E)** *PyMT* mice treated with either IL-4 neutralizing IgG (11B11) or control IgG. ELISA was preformed on conditioned medium from TAMs (50,000) after 18 hours of culture. Quantitative RT-PCR analysis was performed using the comparative threshold cycle method to calculate fold change in gene expression normalized to *GAPDH* as reference gene. Representative assays from 3 independent cohorts each run at least in triplicate are depicted as the mean fold change from the standardized sample.

B-E) Data are represented as mean ± SEM. * denotes $p<0.05$ by Mann-Whitney.

Figure 6. IL-4 signaling promotes metastasis but not primary tumor development.

A) Kaplan Meyer analysis of tumor incidence in *PyMT/IL-4Rα*$^{+/-}$ and *PyMT/IL-4Rα* $^{-/-}$ mice (n=15/group) depicted as % of tumor free animals. No statistical differences between cohorts by generalized Wilcoxon test were found.

B) Total mammary tumor burden of *PyMT/IL-4Rα*$^{+/-}$ *and* $^{-/-}$ mice, and *PyMT* mice treated for 20 days with either IL-4 neutralizing Ig (11B11) or control IgG and evaluated at d100, shown as mm^3 (n= >20 mice per cohort). Tumor size was determined by caliper measurement and multiple tumors in one animal were added together.

C) Quantification of average number of metastatic foci/lung/mouse of 100 day-old *PyMT/IL-4Rα* and *PyMT* mice treated with either IL-4 neutralizing or control IgG. Each lung was serially sectioned and

6 sections 100 μm apart were stained by H&E and total number of metastatic foci (greater then 5 cells) quantified. Each of the six sections was summed and each bar represents n ≥ 23 mice for all cohorts and ≥12 for Ig treated groups.

Figure 7: IL-4 and CSF-1 signaling intersect and regulate macrophage EGF expression, pMEC invasion and metastasis.

A) *EGF* mRNA expression analysis from CSF-1- and IL-4-activated TAMs. TAMs were isolated from mammary tumors of 95 day-old *PyMT/CD4*$^{+/-}$ or *PyMT/CD4*$^{-/-}$ mice, and placed into culture with CSF-1 (10ng/ml) and/or IL-4 (20 ng/ml) for 16 hours. Quantitative RT-PCR analysis of *EGF* mRNA expression is depicted as fold change from vehicle (*CD4*$^{-/-}$ no treatment is set to 1.0) assessed by the comparative threshold cycle method normalized to reference gene expression.

B) Quantification of pMEC organoid disruption following co-culture of TAMs (48 hours) +/- IL-4 (10 ng/ml) or EFGR small molecule inhibitors PD153035 (0.1 μM) or BIBX1382 (10 nM). Invasive organoids were counted and data represented as a percentage of the total organoids (>100 replicate). Representative data from 2 independent experiments performed in quadruplicate are depicted.

C) EGFR signaling regulates metastasis in *PyMT* mice. 110 day-old *PyMT* mice were treated with the EGFR small molecule inhibitor PD153035 (25 mg/kg) versus vehicle (DMSO) by i.p. injection 5 hours prior to analysis (6 mice/group). Presence of circulating carcinoma cells (cytokeratin^{+}CD45^{-}) was assessed by FACS evaluation of live cells in PB.

A-C) Data are represented as mean ± SEM, and * denotes $p<0.05$ by Mann-Whitney.

D) Schematic representation of T_H2 CD4^{+} T lymphocytes and their role in breast cancer metastasis. During early breast cancer development, increased presence of leukocytes in neoplastic stroma indicates establishment of a pro-inflammatory microenvironment. When immune cell infiltrates include high numbers of T_H2 CD4^{+} T lymphocytes that produce IL-4 and IL-13, M2-type TAMs and IMCs are activated that in turn produce EGF, thus resulting in activation of a paracrine-mediated enhancement of malignant cell invasion and dissemination into PB and pulmonary metastasis.

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Figure 1

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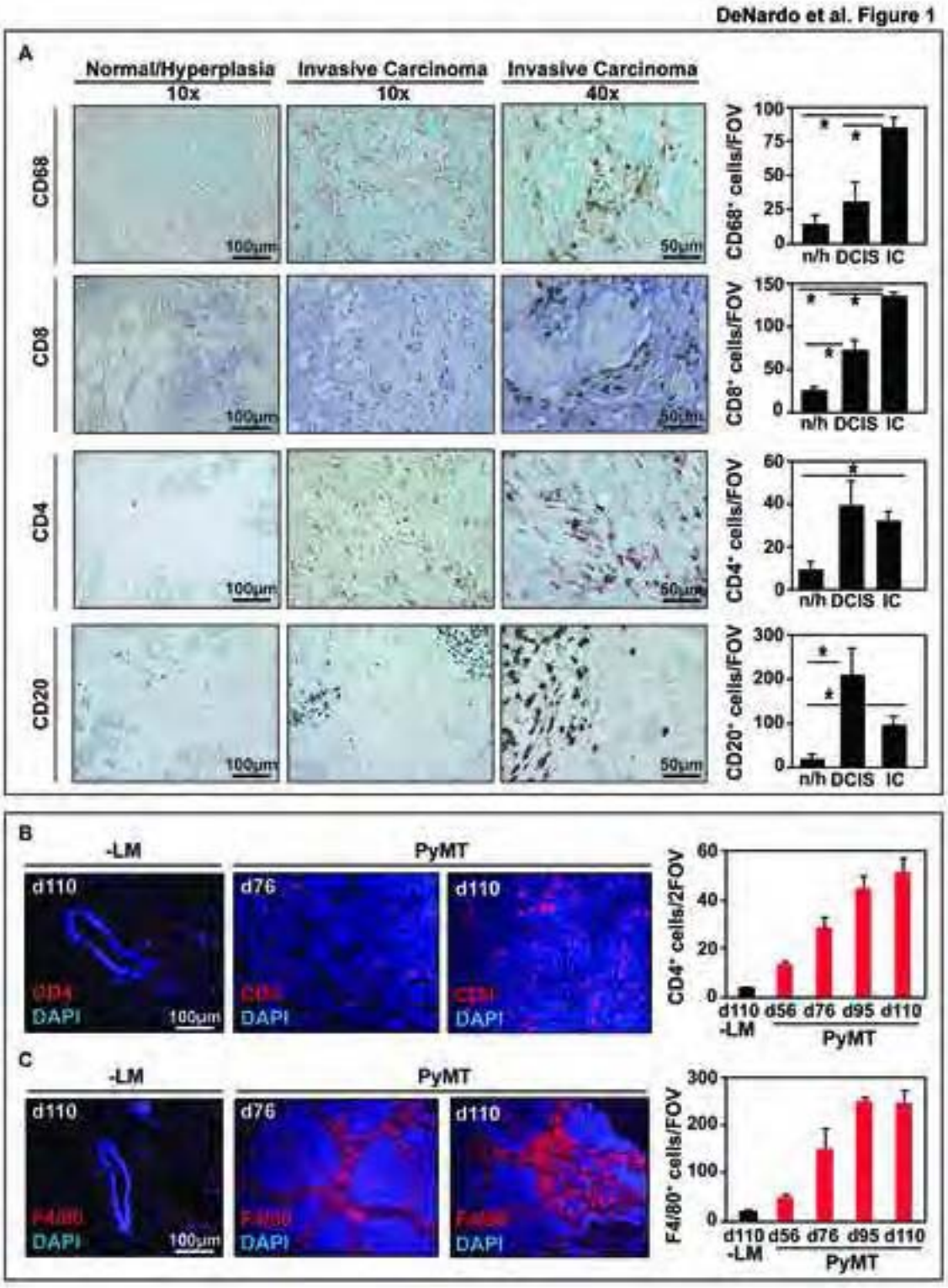

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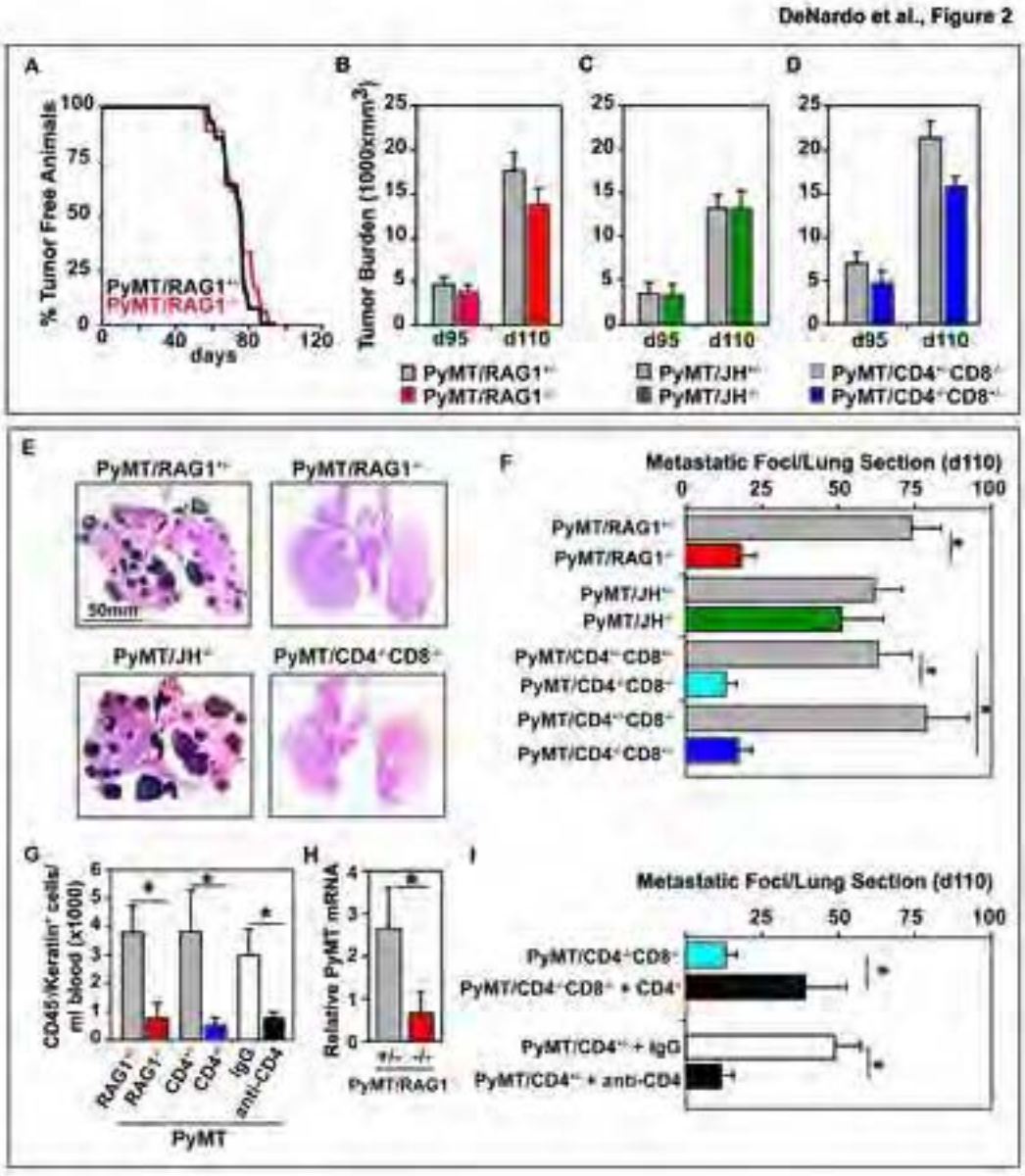

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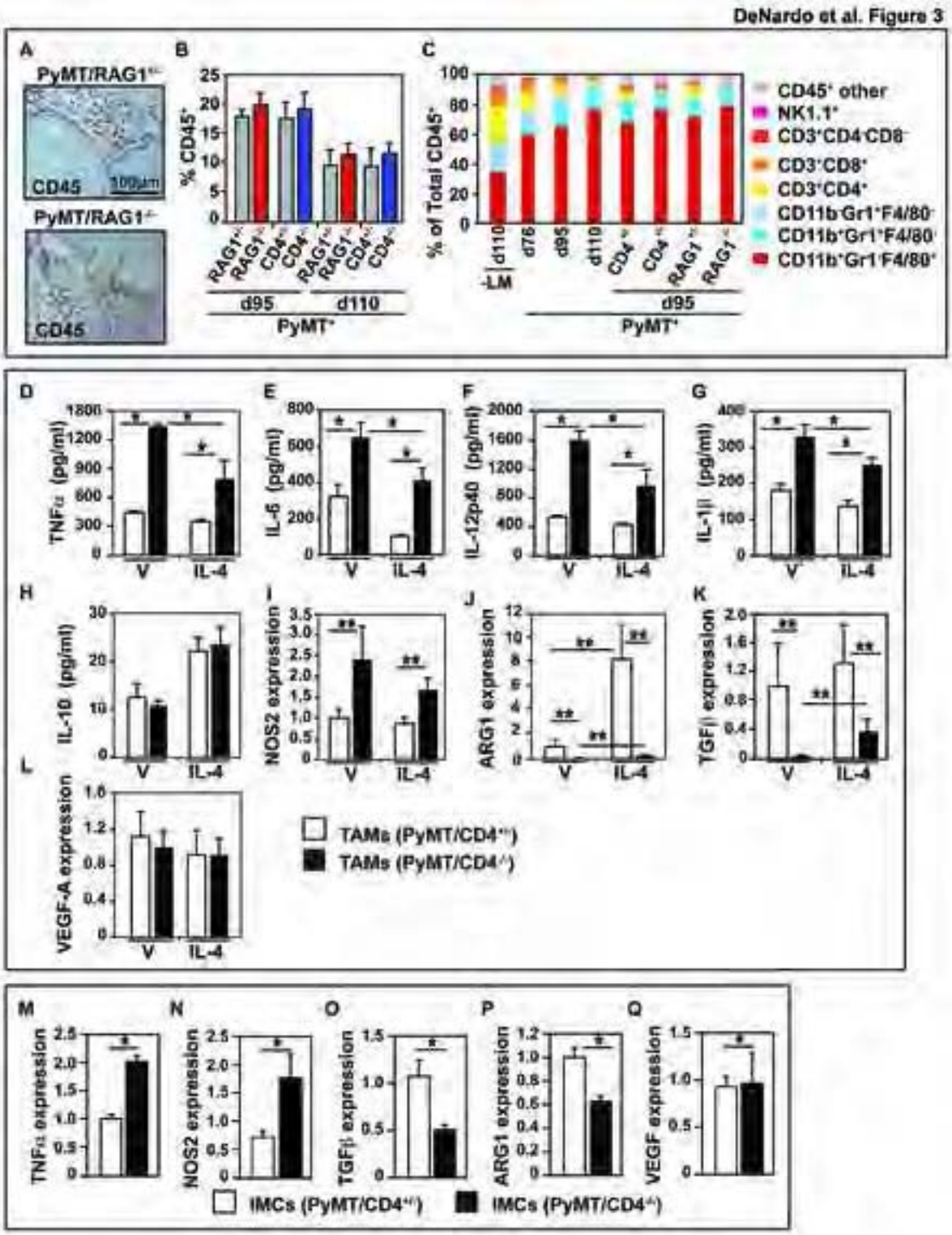

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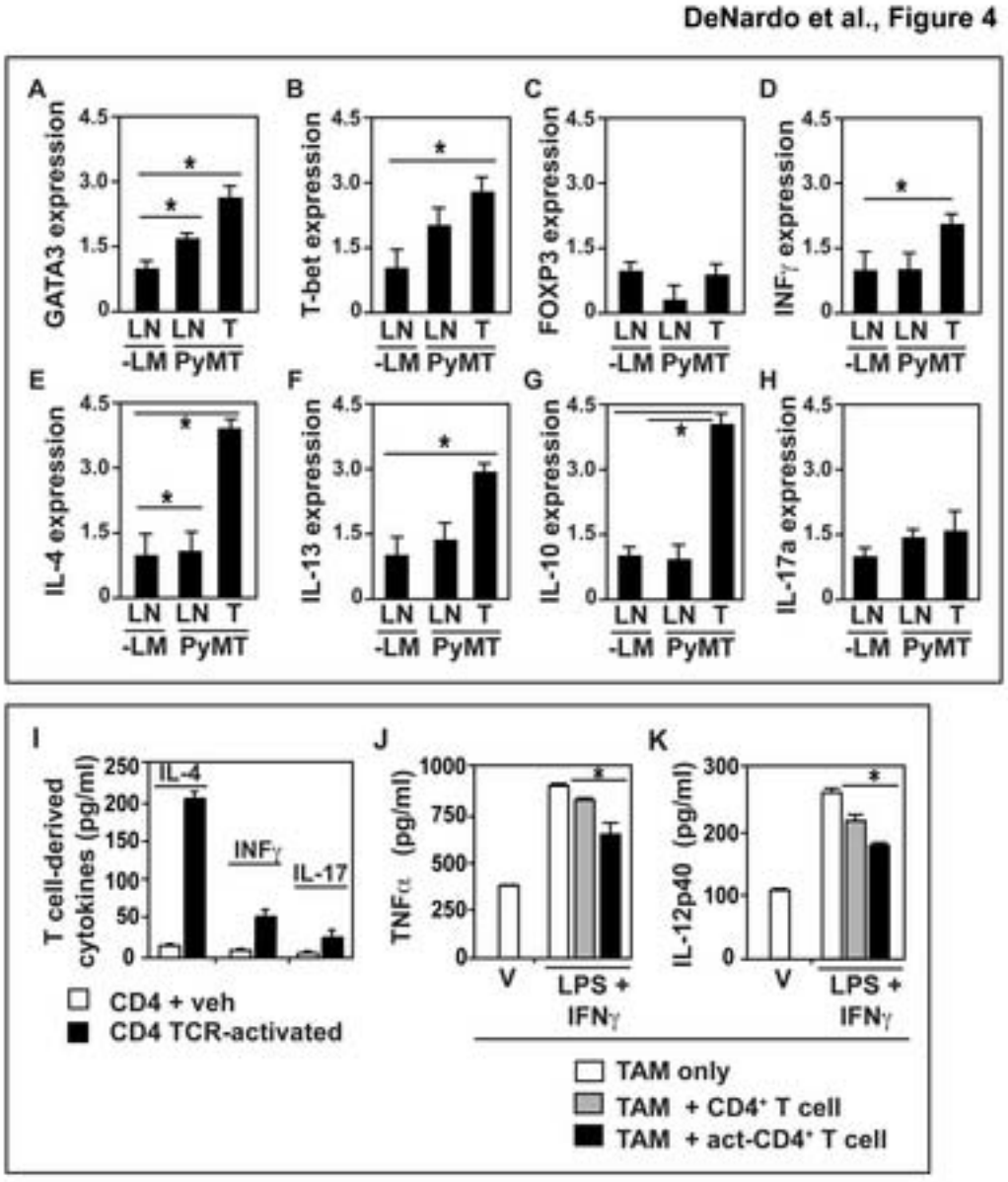

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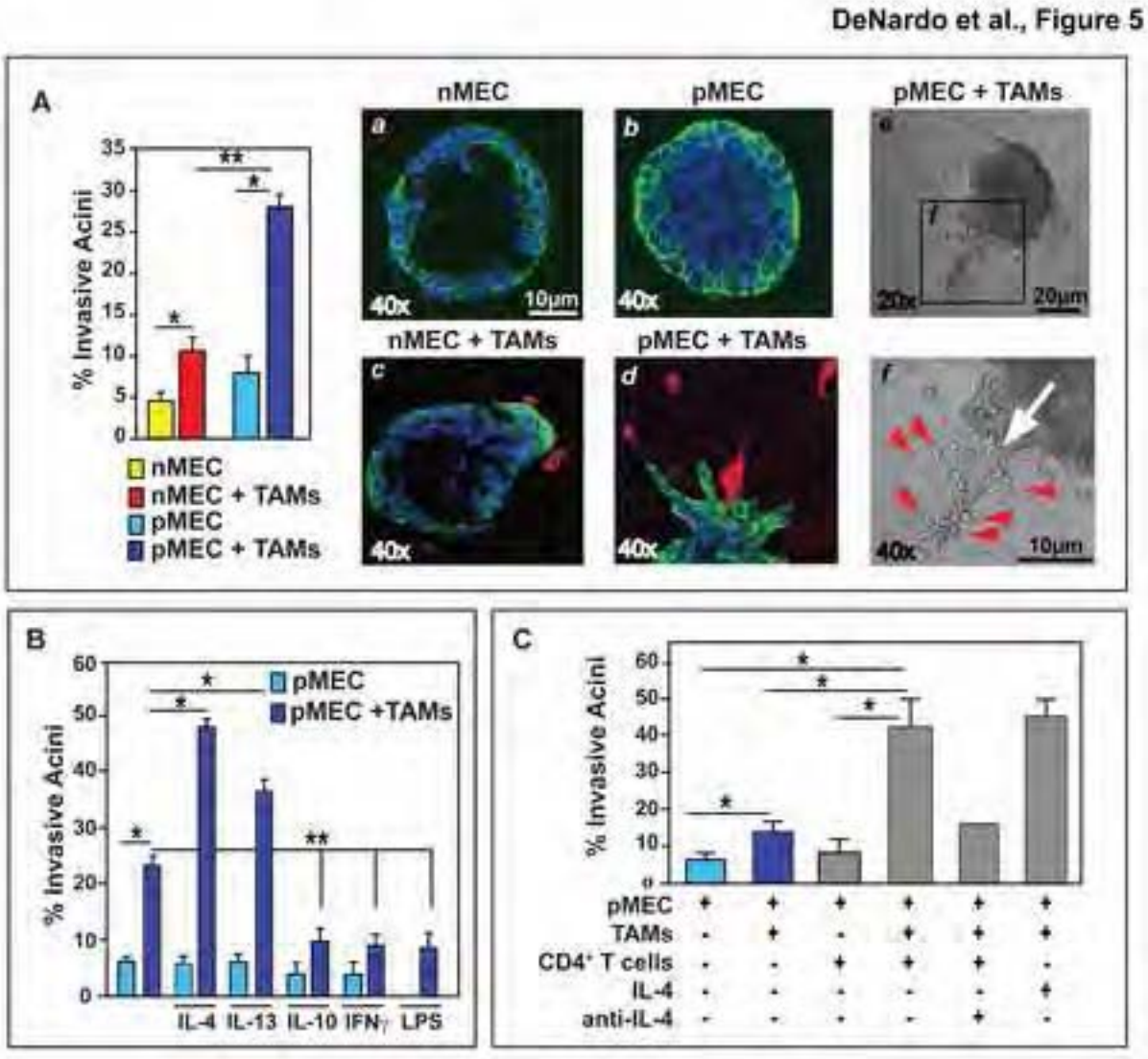

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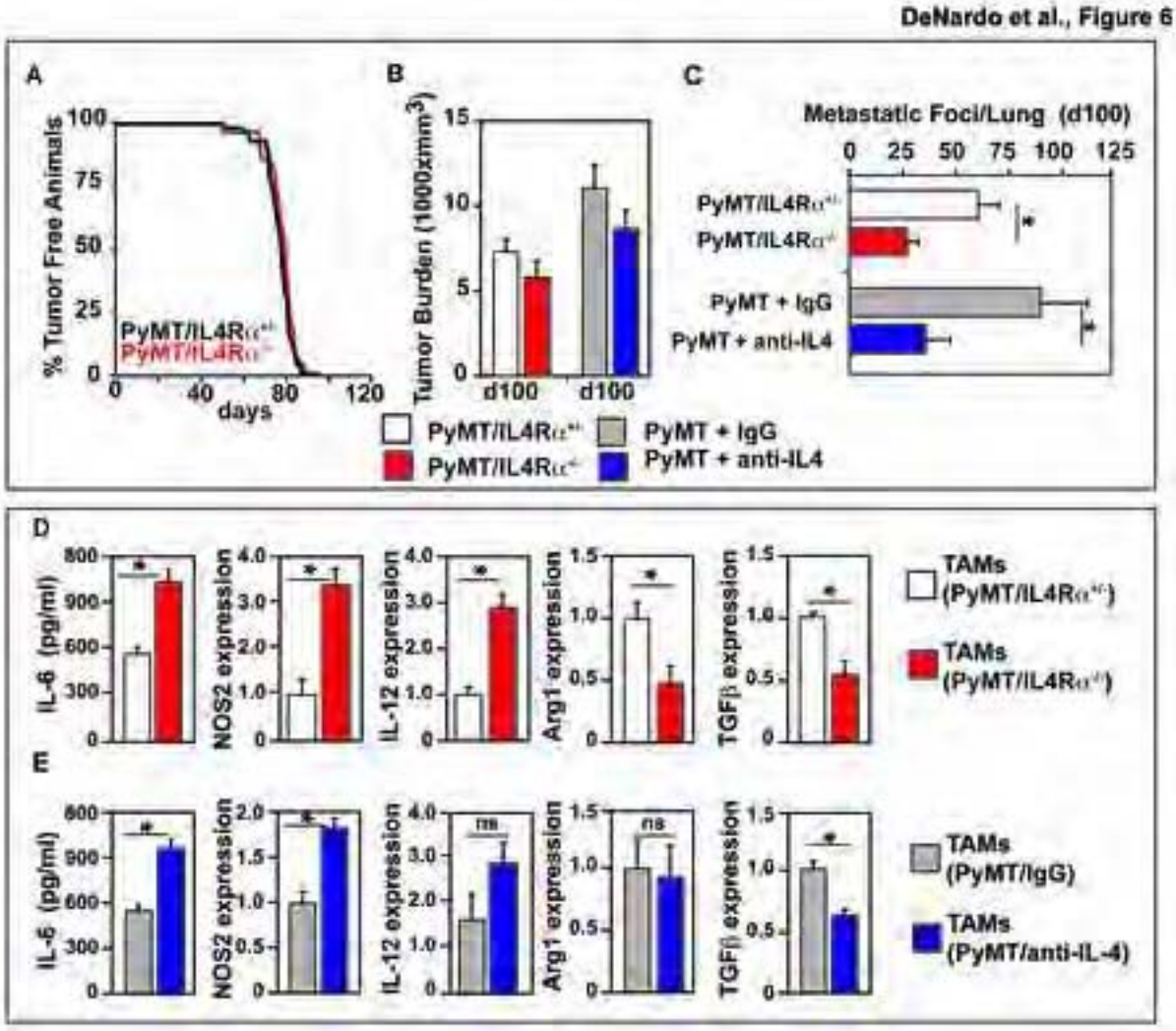

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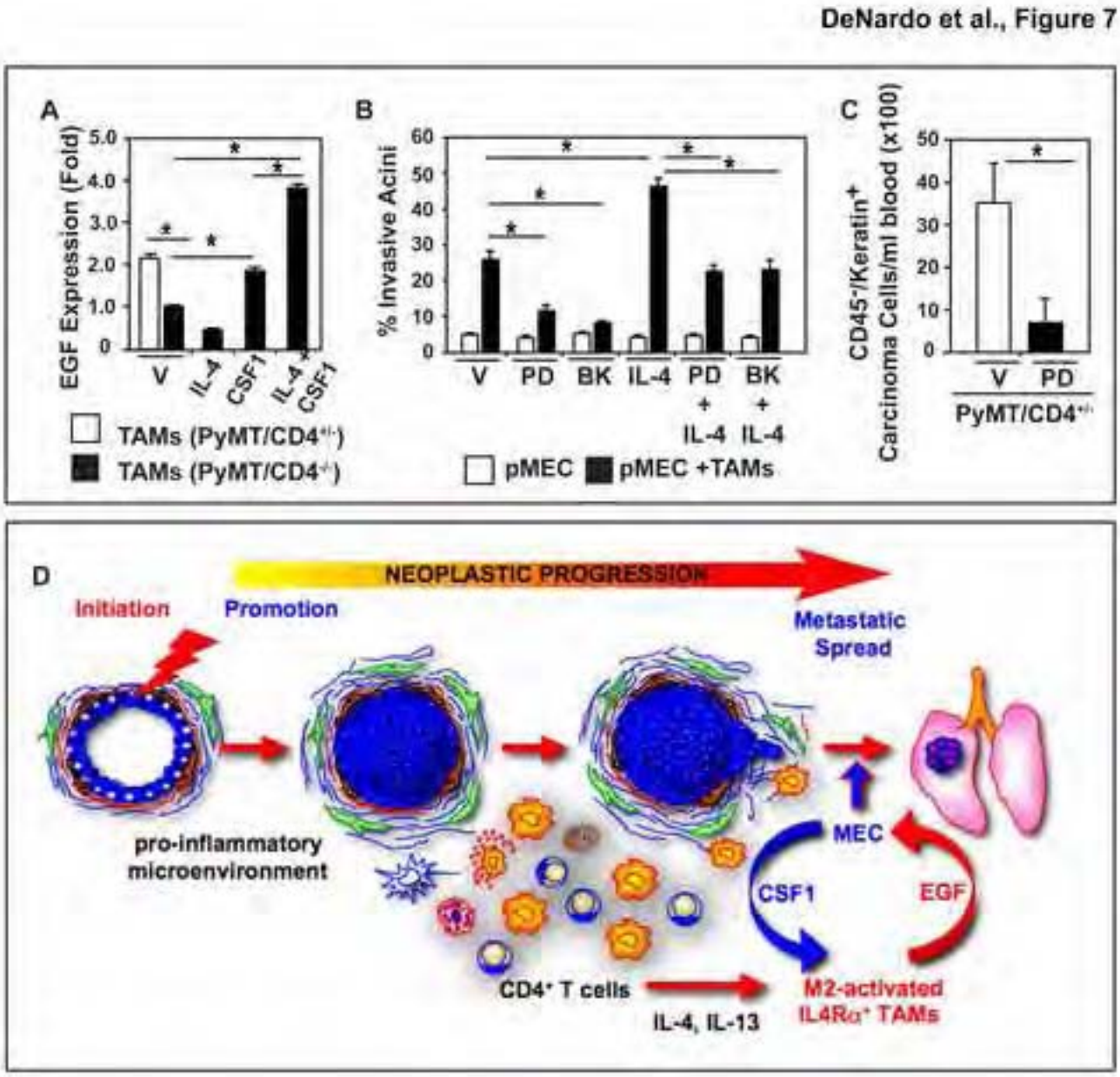

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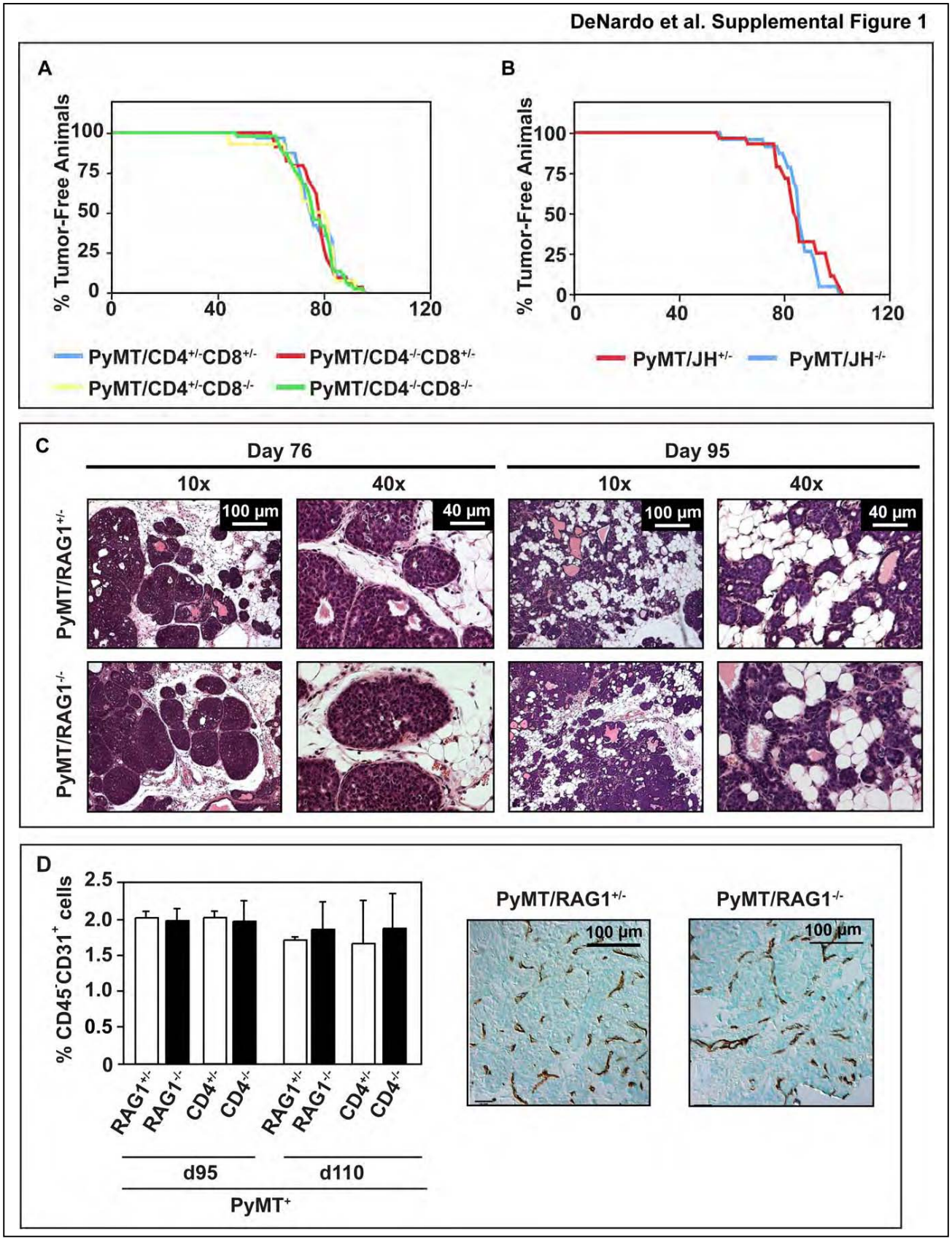
Supplemental Data/Figure Legends/Methods

SUPPLEMENTAL DATA



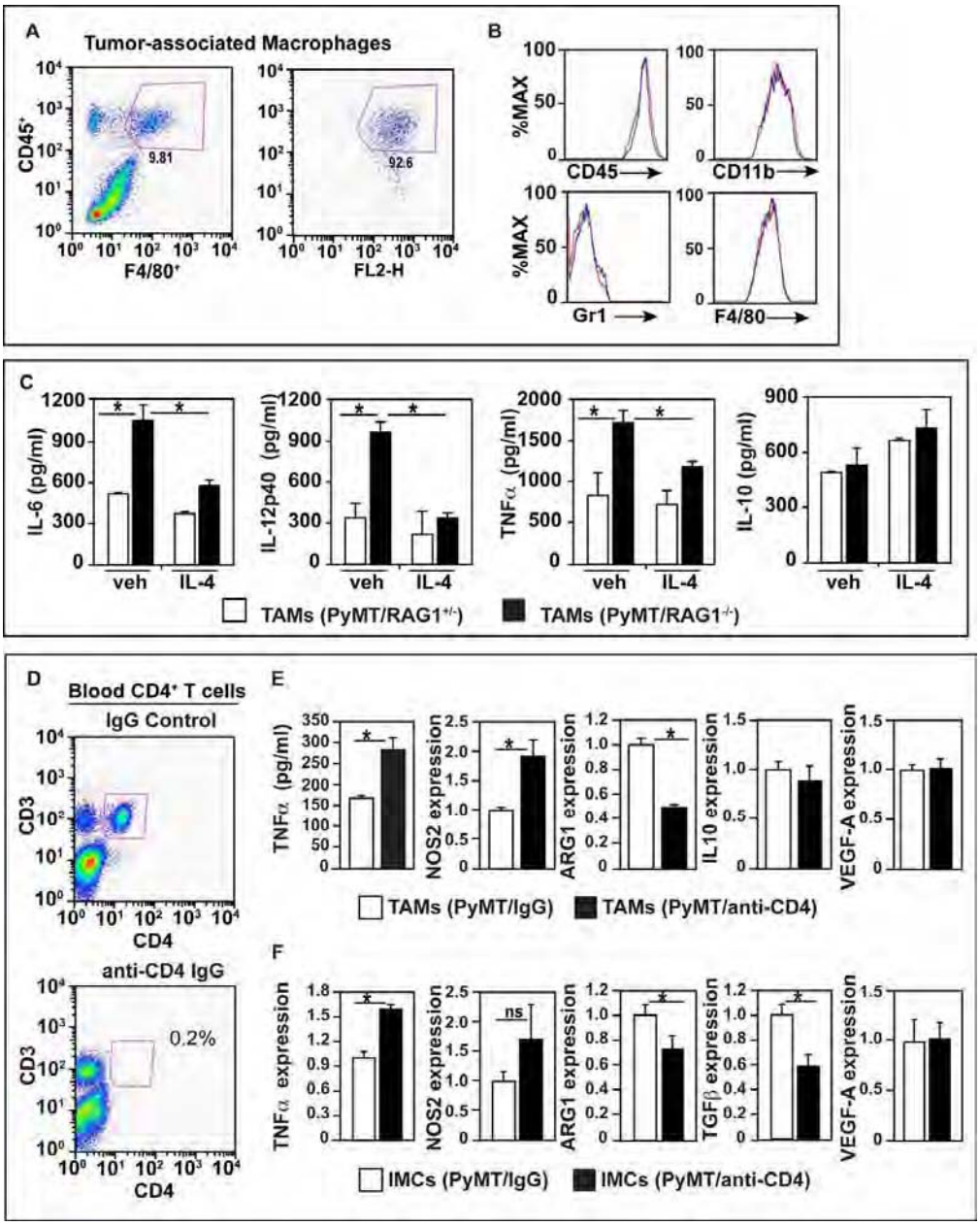
DeNardo et al., 2009 CC-D-08-00948
Supplemental Data/Figure Legends/Methods

Supplemental Figure 1: CD4$^+$ T cells primary tumor development.

A-B) Kaplan Meyer analysis of tumor incidence in CD4, CD8 and B cell-deficient/*PyMT* mice. The percent of tumor free animals is depicted for *PyMT/CD4$^{+/-}$CD8$^{+/-}$* (n=10), *PyMT/CD4$^{-/-}$CD8$^{-/-}$* (n=46), *PyMT/CD4$^{-/-}$CD8$^{+/-}$* (n=34), *PyMT/CD4$^{+/-}$CD8$^{-/-}$* (n=31) as well as *PyMT/JH$^{+/-}$* (n=23) and *PyMT/JH$^{-/-}$* (n=28) mice. Animals were considered to be tumor free until a palpable mass (>4.0 mm) persisted for longer then 4 days. There were no statistical differences between tumor incidence curves by generalized Wilcoxon test.

C) Representative images of H&E stained mammary tumor tissue from 76 and 95-day old *PyMT/RAG1$^{+/-}$* and *PyMT/RAG1$^{-/-}$* mice. Magnification and scale are shown.

D) Flow cytometric analysis of CD31$^+$ endothelial cells in mammary tumors from 95 and 110 day-old *PyMT/RAG1$^{+/-}$*, *PyMT/RAG1$^{-/-}$*, *PyMT/CD4$^{+/-}$CD8$^{+/-}$*, *PyMT/CD4$^{-/-}$CD8$^{-/-}$* mice (n=4). Data is depicted as the mean % CD31$^+$ cells of the total live cells/tumor ±SEM. No statistical differences were found between groups by Mann-Whitney test. Representative 20x images of paraffin embedded tumor sections from *PyMT/CD4$^{+/-}$*, *PyMT/CD4$^{-/-}$* sections stained for CD31$^+$ and vasculature revealed as brown staining. Bar in both = 100 μm.



Supplemental Figure 2: Immune cell infiltration and endothelial expansion in the absence of adaptive immunity.

A-B) Isolation of TAMs from the mammary tumors of *PyMT/CD4*$^{+/-}$ and *PyMT/CD4*$^{-/-}$ mice. The purity of isolated CD45^{+}F4/80^{+}Gr1^{-} macrophages was assessed by flow cytometric analysis of the cell suspension before and after selection. Representative analyses are shown from *PyMT/CD4*$^{+/-}$(red) and *PyMT/*CD4$^{-/-}$ (blue) tumors and relative CD45, CD11b, Gr1 and F4/80 expression depicted.

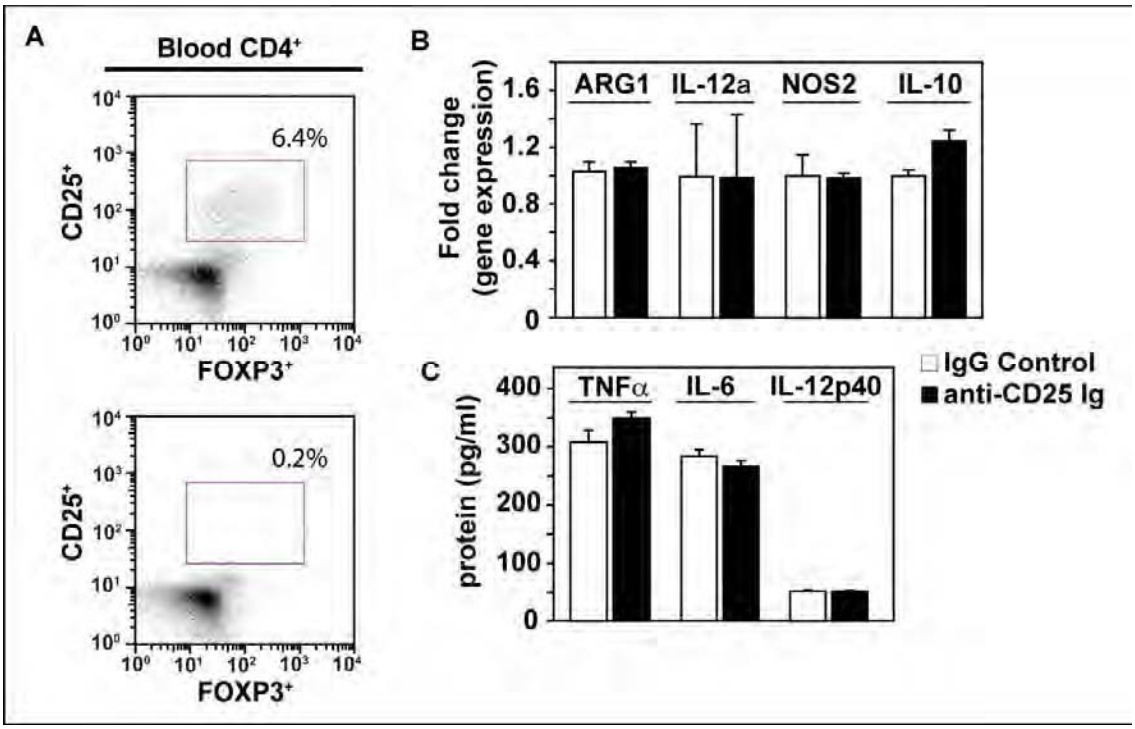
C) Cytokine expression analysis from TAM-conditioned medium. Tumor-associated CD45^{+}F4/80^{+}Gr1^{-}

macrophages were isolated by dual magnetic and flow sorting of mammary tumors from 95 day-old *PyMT/RAG1*$^{+/-}$ and *PyMT/RAG1*$^{-/-}$ mice (n=3/cohort). Cytokine expression was assessed by ELISA of conditioned medium from TAMs (25,000) following 18 hours of culture, with or without recombinant IL-4 (10 ng/ml). Representative assays from 2 independent cohorts each performed at least in triplicate are depicted as means ± SEM and * denotes $p<0.05$ by student t test.

D) Lymphocyte depletion by anti-CD4 IgG (GK1.1) was assessed by flow cytometric analysis 4 days after of IgG injection. Representative analyses from anti-CD4 IgG and control IgG treated mice are shown from blood isolated by left ventricle heart puncture. % $CD4^+$ of total cells is depicted.

E) Analysis of TAM polarization following $CD4^+$ T cells depletion. Tumor-associated $CD45^+F4/80^+Gr1^-$ macrophages were isolated by flow sorting mammary tumors from *PyMT* mice treated with either anti-CD4 IgG (GK1.1) or control IgG. Tumor bearing 85 day-old mice were treated twice over 10 days with Igs and TAMs were isolated from tumors when mice were 95 day-old. Cytokine expression was assessed by ELISA analysis of conditioned medium from TAMs following 18 hours of culture. Gene expression analysis used the comparative threshold cycle method was used to calculate fold change in gene expression normalized to *GAPDH* as reference gene. Representative assays from 2 independent cohorts each run at least in triplicate are represented as mean values ± SEM. * denotes n $p<0.05$ by Mann-Whitney.

F) Analysis of IMC polarization following $CD4^+$ T cells depletion. Tumor-associated $CD45^+CD11b^+Gr1^{Hi}$ IMCs were isolated by flow sorting mammary tumors from *PyMT* mice treated with either anti-CD4 IgG (GK1.1) or control IgG. Tumor bearing 85 day-old mice were treated twice over 10 days with Igs and TAMs were isolated from tumors when mice were 95 day-old. Cytokine expression was assessed by ELISA and quantitative real-time PCR as described above. Representative assays from 2 independent cohorts each run at least in triplicate are represented as mean values ± SEM. * denotes $p<0.05$ by Mann-Whitney.



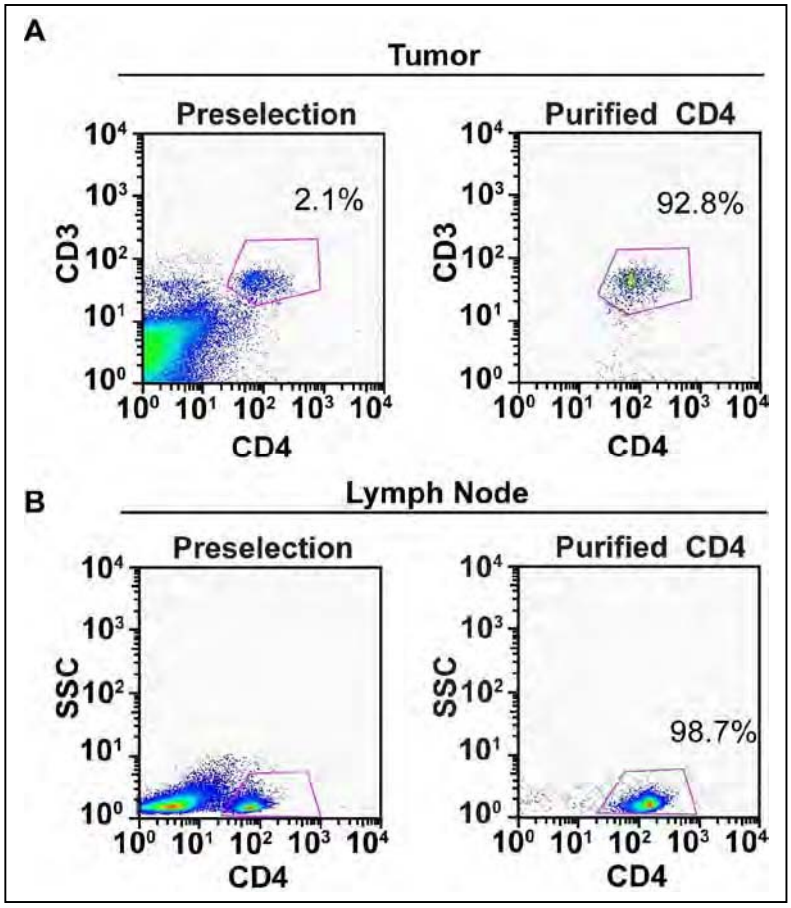
Supplemental Figure 3: Cytokine profiles of TAMs from CD25 T_{reg}-depleted mice.

A) Analysis of $CD25^+$ T cells depletion. Tumor bearing 85 day-old *PyMT* mice (5 mice/cohort) were treated with either anti-CD25 IgG (PC61) or control IgG three times over 15 days. Peripheral blood was analyzed for expression of FOXP3 and CD25 expression on $CD4^+$ T lymphocytes every 5 days by flow cytometry to affirm depletion. Representative analysis for depleted versus control 100 day-old *PyMT* mice is depicted as FACS plots gated on $CD4^+$ lymphocytes.

B) Quantitative real-time PCR analysis of *Nos2, Arg1, Tgfβ, IL-12a* and *IL-10* mRNAs expression in tumor-associated $CD45^+F4/80^+Gr1^-$ macrophages isolated by flow sorting from mammary tumors of 100 day-old *PyMT* mice treated with either anti-CD25 Ig (PC61) or isotype control IgG. The comparative threshold cycle method was used to calculate fold change in gene expression normalized to *GAPDH* as reference gene. Cell isolation and gene expression analysis was performed on 2 separate cohorts of animals (3 mice/group) with similar results. Representative data are depicted as means ± SEM. No statistical differences were found between treated and untreated groups by Mann-Whitney.

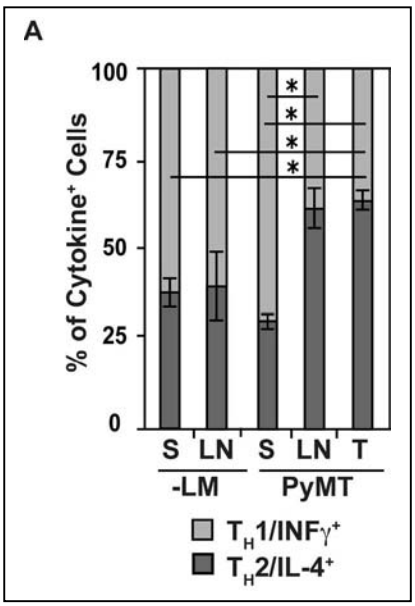
C) Cytokine expression analysis from TAM conditioned medium. Tumor-associated $CD45^+F4/80^+Gr1^-$ macrophages were isolated by flow sorting from mammary tumors from 100 day-old *PyMT* mice treated with either anti-CD25 Ig (PC61) or isotype control IgG for 15 days. Cytokine

expression was assessed by ELISA analysis of conditioned medium from TAMs following 18 hours of culture. Cell isolation and cytokine analysis was performed on 3 separate cohorts of animals (2 mice/group) with similar results. Representative data are depicted as mean ± SEM. No statistical differences were found between treated and untreated groups by Mann-Whitney.



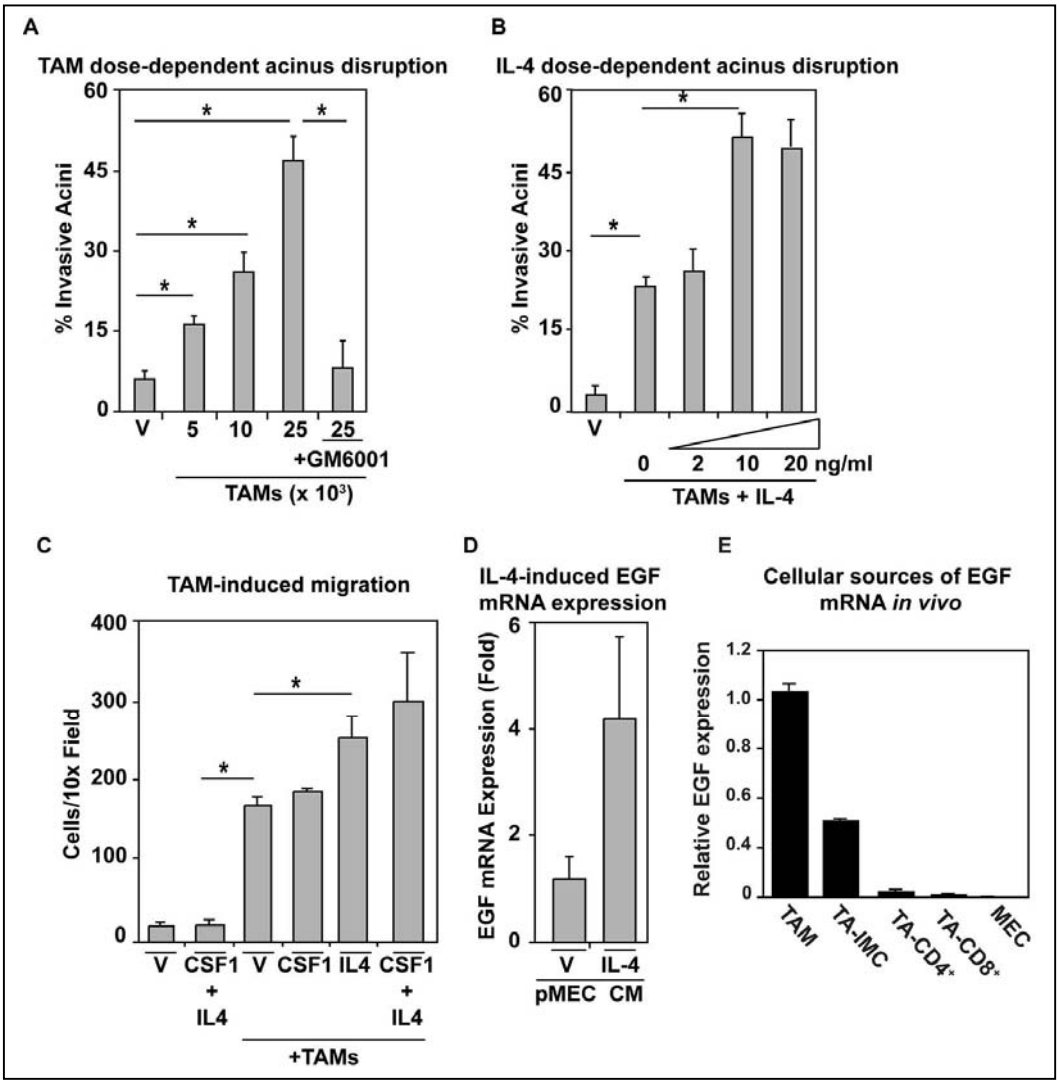
Supplemental Figure 4. CD4 T lymphocyte purification and depletion.

A-B) Isolation of $CD4^+$ T lymphocytes from tumors and lymph nodes. Selection was done by magnetic selection for CD4 followed by FACS sorting for CD3 and CD4 positive cells. The purity of isolated cells was assessed by flow cytometric analysis of pre-selection and purified cells. Representative analyses are shown from tumor and LN isolations. % $CD4^+$ of total cells is shown.



Supplemental Figure 5.

A) Analysis of intracellular cytokine expression by $CD4^+$ T cells. $CD4^+$ T cells were isolated by flow sorting from spleen (S), lymph nodes (LN) or tumors (T) from 95 day-old (-)LN or *PyMT* mice (n=3/cohort). Isolated $CD4^+$ T cells were activated *ex vivo* by treatment with anti-mouse CD3 and CD28 IgGs in the presence of Brefeldin A. Cells were fixed and intracellular IL-4 and IFNγ was calculated after 6 hours of activation. The Ratio of $IL\text{-}4^+$ to $IFN\gamma^+$ $CD4^+$ T cells is depicted as mean % of cytokine positive cells from 3 independent isolation experiments and * denotes $p<0.05$ by Mann-Whitney.



Supplemental Figure 6: Alternatively activated TAMs induce malignant behavior.

A) Quantification of pMEC organoid disruption following co-culture with TAMs (isolated from mammary tumors of 95 day-old *PyMT* mice). Metalloprotease activity was inhibited by treatment with a broad-spectrum MMP inhibitor GM6001 (10 μM). Disrupted organoids were quantified and data represented as a percentage of total organoids (>100 replicate/4 replicates). Data depicted as mean ± SEM and * denotes $p<0.05$ by Mann-Whitney.

B) Quantification of organoid disruption following co-culture of TAM (48 hours) with pMEC, in the presence of either vehicle (V) or increasing concentrations of IL-4 (2-20 ng/ml). Disrupted organoids were counted and then expressed as a percentage of the total organoids present. Disrupted organoids were counted and data represented as a percentage of the total organoids (>100 replicate/4 replicates).

Data depicted as mean ± SEM and * denotes $p<0.05$ by Mann-Whitney.

C) Quantification of pMEC migration/chemotaxis in response to TAMs treated with IL-4 (10 ng/ml) and/or CSF-1 (10ng/ml) assessed using a Boyden chamber assay. The number of pMECs that migrated to the opposite side of the membrane was assessed by H&E staining, four 10x fields were quantified per membrane (using Image J) and 4 inserts were used for each condition. Data are represented as mean ± SEM and * denotes $p<0.05$ by Mann-Whitney.

D) Quantitative real-time PCR analysis for *EGF* mRNA expression from $CD45^+F4/80^+Gr1^-$ macrophages isolated by flow sorting from mammary tumors from 95 day-old *PyMT/CD4*$^{+/-}$ mice. TAMs were treated pMEC conditioned medium (24 hours conditioning) with or without IL-4 (20 ng/ml). The comparative threshold cycle method was used to calculate fold change in gene expression normalized to *β-actin* as a gene reference. Representative data from 2 independent experiments is depicted as the mean fold change from the standardized sample ± SEM. In all panels, * denotes $p<0.05$ by Mann-Whitney.

D) Macrophages as a source of EGF in mammary carcinomas. Quantitative real-time PCR analysis of *egf* mRNA expression in tumor-associated $CD45^+F4/80^+Gr1^-$ macrophages (TAMs), $CD45^+CD11b^+Gr1^{HI}$ IMCs, $CD4^+$ and $CD8^+$ T cells as well as MECs from mammary carcinomas of 100 day-old *PyMT* mice isolated by flow sorting. The comparative threshold cycle method was used to calculate fold change in gene expression normalized to *GAPDH* as reference gene. Cell isolation and gene expression analysis was performed on 4 separate cohorts of animals (1-2 mice/cohort). Representative data are depicted as mean ± SEM.

SUPPLEMENTAL METHODS:

ELISA

TNFα, IL-6, IL-1b, IL-12, IL-4, IL-17 and IFNγ concentrations of conditioned medium were assayed using Ready-Set-Go ELISA kits (eBioscience) and IL-10 assessed using a quantikine immunoassay (BD Bioscience) as described by the manufacturer. Optical density was measured at 450 nm with wavelength correction set to 540 nm on a SpectraMax 340 spectrophotomoter (Molecular Devices).

Quantitative RT-PCR

Total RNA was extracted from 200-300,000 FACS sorted $CD45^{+}F4/80^{+}Gr1^{-}$ cells using an RNeasy Mini Kit (QIAGEN). cDNAs were synthesized using Superscript III^{tm} First-strand synthesis (Invitrogen). Primers specific for *β-actin, GAPDH EGF, Vegf-a, MMP-9, Tgfβ, Arignase-1, and Nos2* (Superarray) were used and relative gene expression was determined using RT^2 Real-Time^{tm} SYBR Green/ROX PCR master mix (Superarray) on an ABI 7900HT quantitative PCR machine (ABI biosystems). The comparative threshold cycle method was used to calculate fold change in gene expression, which was normalized to both *β-actin* and *Gapdh* as a gene reference. Samples were assayed from at least three independent experiments per category.

Flow cytometric analysis

Single-cell suspensions were prepared from mammary gland dissection by manual mincing using scalpel followed by enzymatic digestion for 40 min at 37°C by Collagenase A 3.0 mg/ml (Roche) and DNase I (Roche) dissolved in DMEM (Invitrogen), under stirring conditions. Digestion mixtures were quenched by adding DMEM containing 10% FBS and then filtered through 0.7 μm nylon strainers (Falcon). Cells were then incubated for 10 min at 4°C with rat anti-mouse CD16/CD32 mAb (BD Biosciences) at a 1:100 dilution in PBS containing 1% of BSA (Sigma) to prevent nonspecific antibody binding. Subsequently, cells were washed twice in PBS/BSA and incubated for 20 min with primary antibody (1:100) followed by two washes with PBS/BSA. 7-AAD (BD Biosciences) was added (1:10) to discriminate between viable and dead cells. Data acquisition and analysis were performed on a FACSCalibur using CellQuestPro software (BD Biosciences).

Tumor and metastatic burden

Tumor burden was determined by caliper measurements on live sedated mice at day 95 and day 110.

Metastatic disease was assessed by serial sectioning of formalin-fixed paraffin-embedded lungs. Entire lungs were sectioned and number of metastatic foci (>5 cells) counted on 6 sections taken every 100 μm following staining with hematoxylin and eosin (H&E). 15 to 30 lungs were analyzed for each cohort indicated.

Immunohistochemistry

Paraffin-embedded tissue sections were fixed in 10 % formalin and incubated with detection antibodies as previously described (Junankar et al., 2006). A biotinylated secondary antibody was applied, followed by incubation with streptavidin-conjugated HRP. Peroxidase activity was localized with diaminobenzidine (Vectastain ABC kit, Vector Laboratories). For immunofluorescent staining Alexa fluor 594 conjugated goat anti-rat (Molecular Probes, 10 mg/ml) was used. All immuno-localization experiments were repeated on multiple tissue sections and included negative controls for determination of background staining, which was negligible. Quantitative analysis of $CD4^+$ and $F480^+$ cells was performed by counting cells in ten high-power fields (20×) per age-matched tissue section from five mice per group. IHC analysis of human breast tissue was accomplished using commercially available tissue microarrays (Pantomics BRC150101, 2, 3). Citrate antigen retrieval (BioGenex) was used for CD4 and CD8 staining and Proteinase XXV (Lab Vision) for CD68. Following blocking in Goat serum, Ab dilutions of 1:25 CD4 (4B12, Novocastra), 1:100 CD68 (KP1, Neomarkers) and CD8 (C8/144B, Neomarkers) were then applied. Appropriate biotinylated secondary antibody was applied, followed by incubation with streptavidin-conjugated HRP. Peroxidase activity was localized with diaminobenzidine (Vectastain ABC kit, Vector Laboratories). The mean number of positive cells in tissue section was evaluated by counting all high power fields (20x) per tissue section (1.1 mm)/ 2 sections/patient using Image J (NIH).

Immune cell isolation

Immune cells were isolated from tumors using a dual purification strategy including magnetic purification followed by flow sorting. Single cell suspensions from tumors were created as described above. Alternatively, single cell suspensions generated from lymph nodes or spleens were passed through 0.7 μm nylon strainers (Falcon). Cells were then incubated for 10 min at 4°C with rat anti-mouse CD16/CD32 mAb (BD Biosciences) at a 1:100 dilution in PBS/BSA then washed twice in PBS/BSA and incubated for 20 min with appropriate fluorescent primary antibodies which included

anti-CD45-APC (30-F11), in addition to anti-CD4 (GK1.1), -CD3 (145-2C11), -Gr-1 (RB6-8G5), -CD11b (93) and/or F4/80 (BM8) (all from eBiosciences) at 1:100 dilution depending on the population to be isolated. Total leukocytes were isolated using magnetic bead selection for APC^+ according to manufactures specifications (Miltenyi Biotec). Magnetically selected cells were then flow sorted on a FACSAria using CellQuestPro software (BD Biosciences).

T cell activation

Dual magnetic and flow-sorted $CD45^+CD3^+CD4^+$ T lymphocytes were added to CD3/CD28 coated plates (5.0 μg/ml, eBioscience). Golgistop (BD Bioscience) was added to the medium and 8 hours later, conditioned medium was isolated and cells stained for CD4 using APC-conjugated anti-mouse CD4 (eBioscience) and then intracellularly with PE-conjugated anti-mouse IFNγ or FITC-conjugated anti-mouse IL-4 using the Cytofix/Cytoperm kit (BD Biosciences).

Supplemental References:

Junankar, S., Eichten, A., Kramer, A., de Visser, K. E., and Coussens, L. M. (2006). Analysis of immune cell infiltrates during squamous carcinoma development. J Invest Dermatology *126 Suppl*, 36-43.

Supplemental Figure 1

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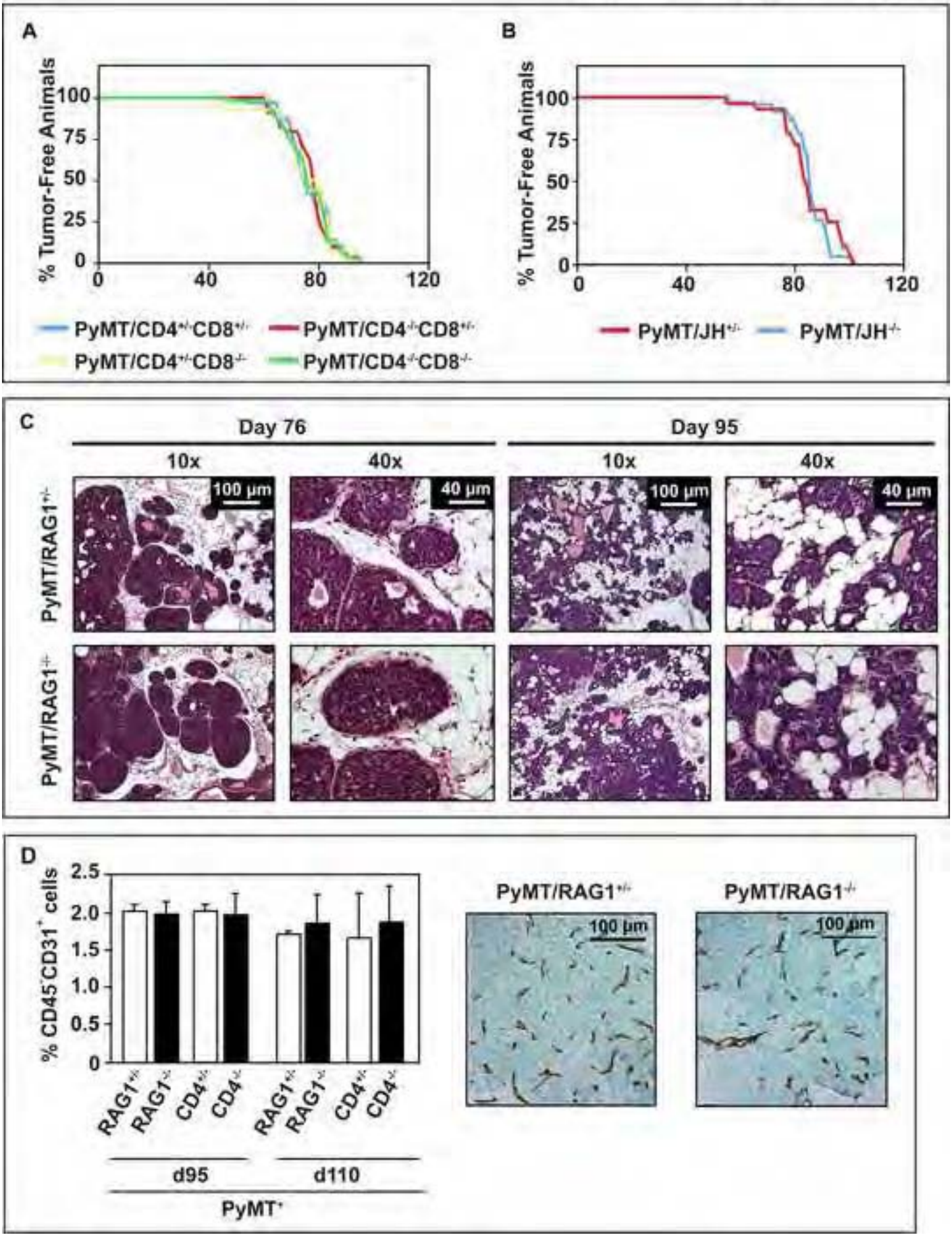
Appendix B

DeNardo et al. Supplemental Figure 1



Supplemental Figure 2

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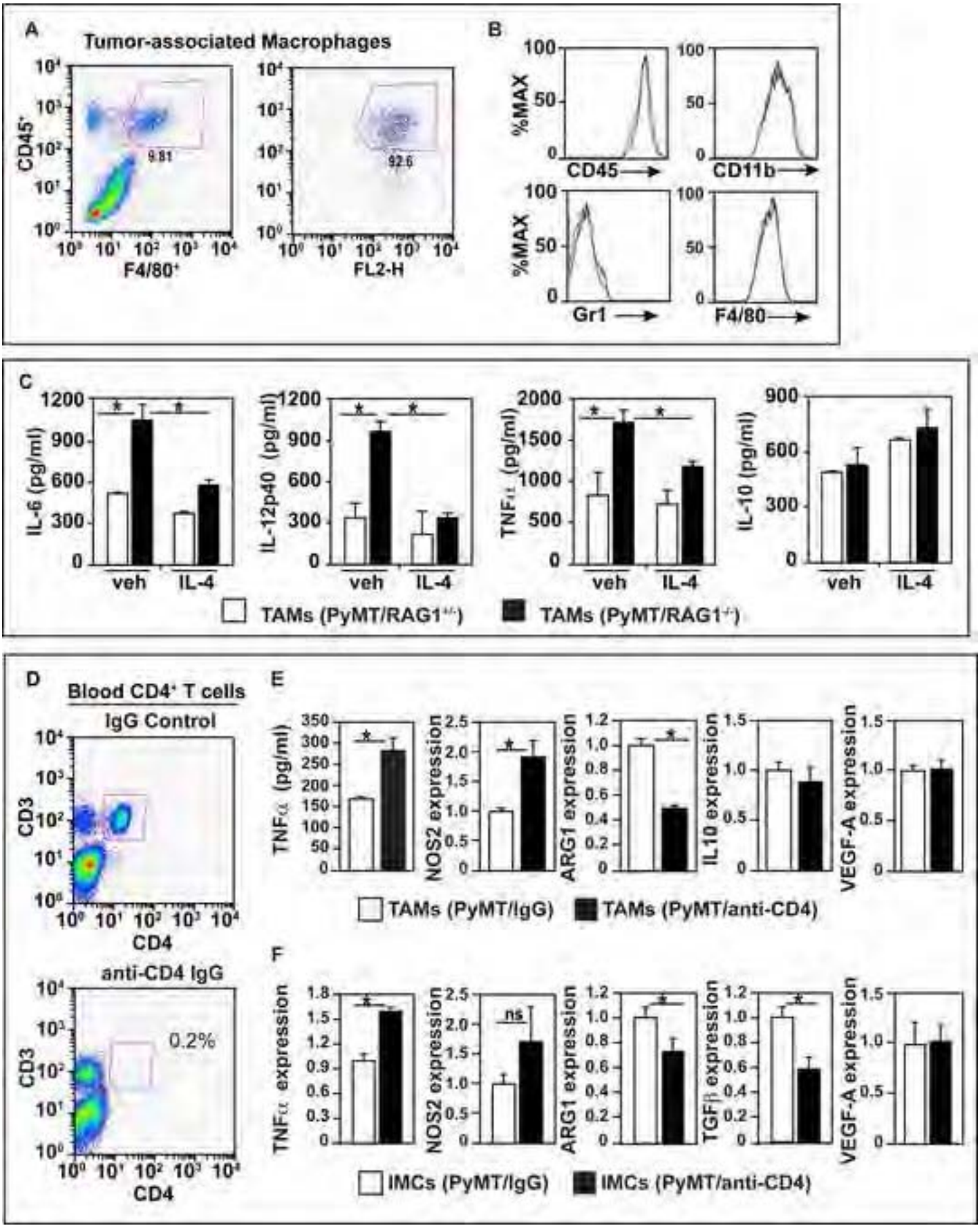

Supplemental Figure 3

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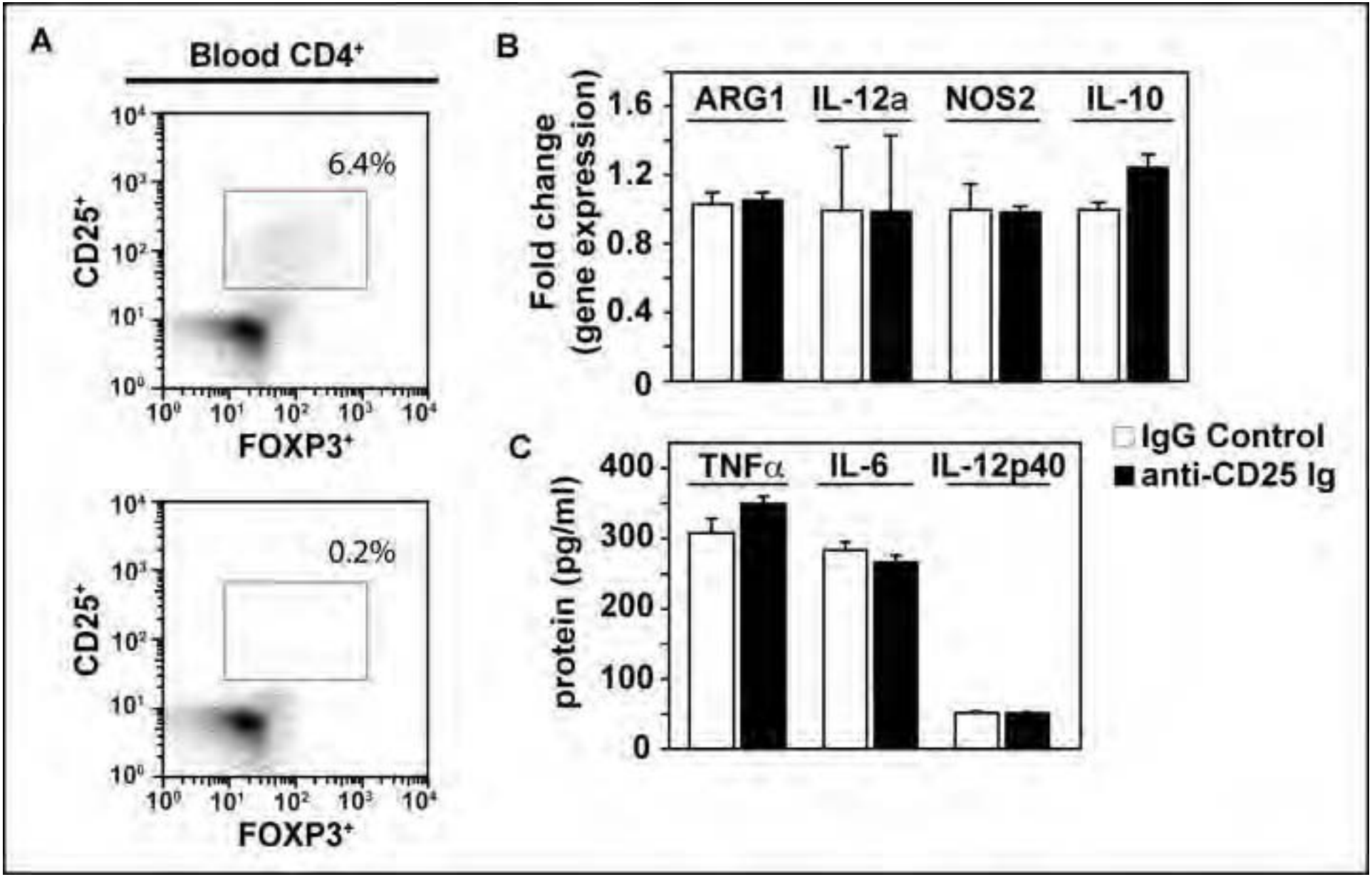

Supplemental Figure 4

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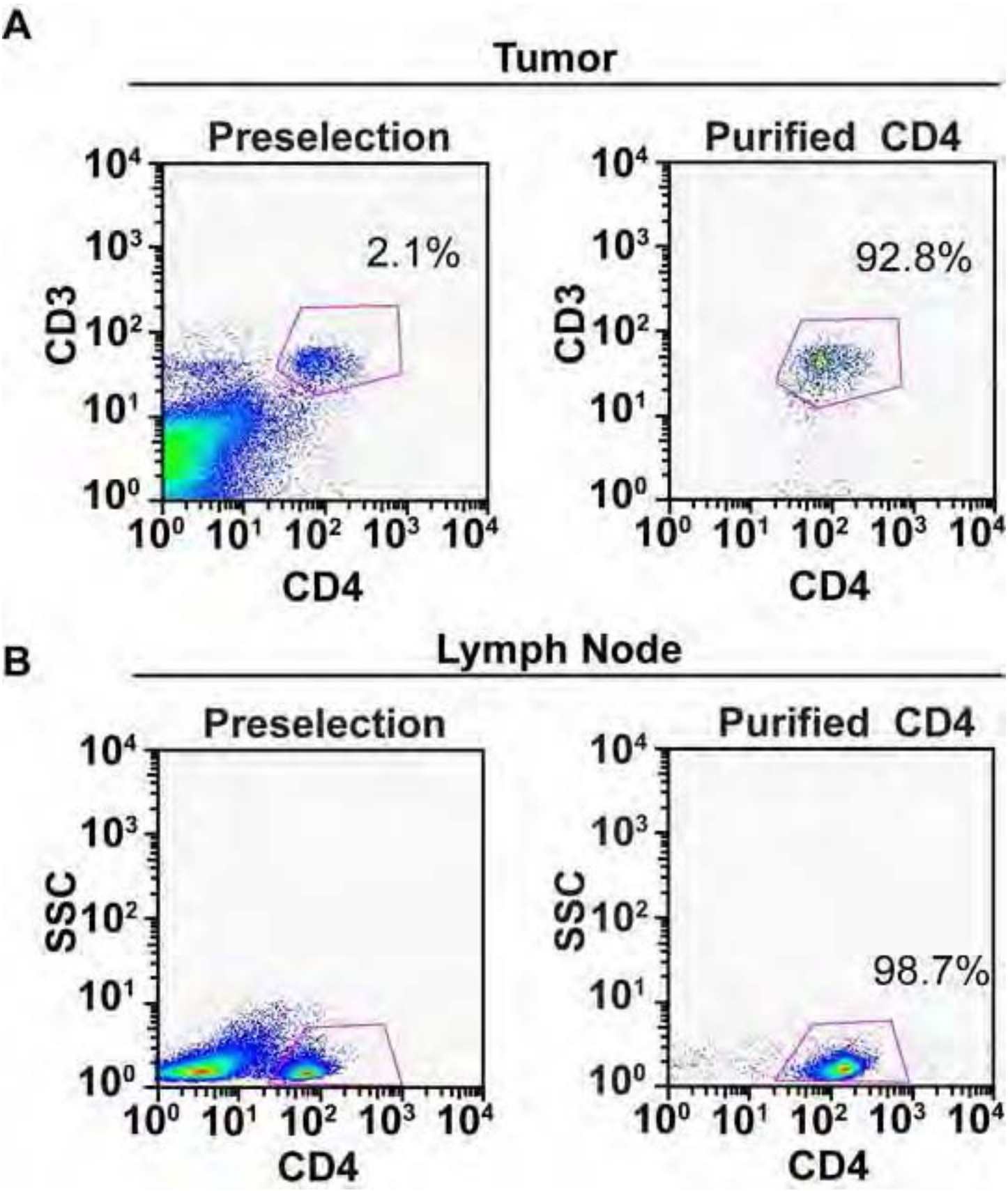

Supplemental Figure 5

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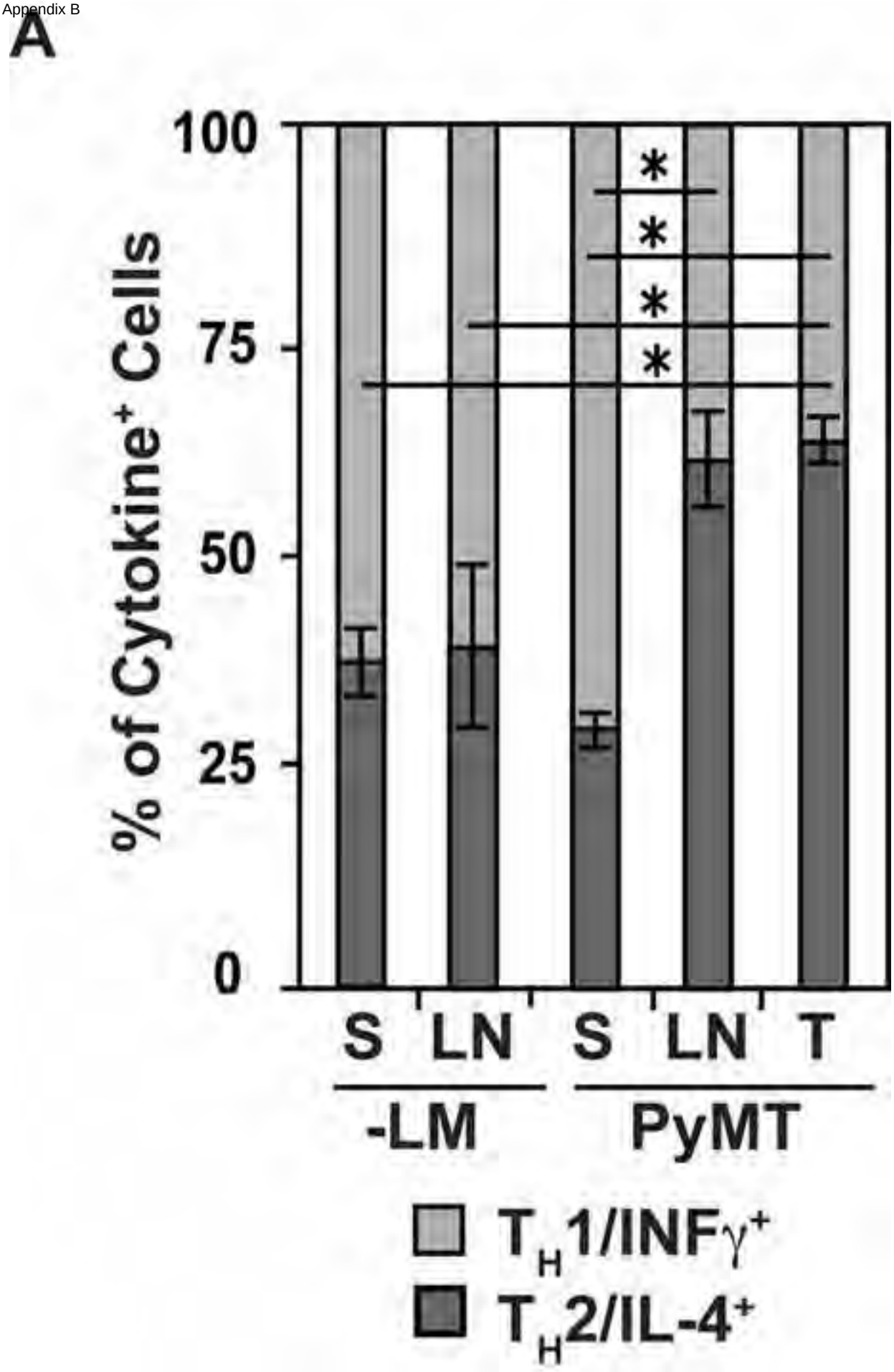

Supplemental Figure 6

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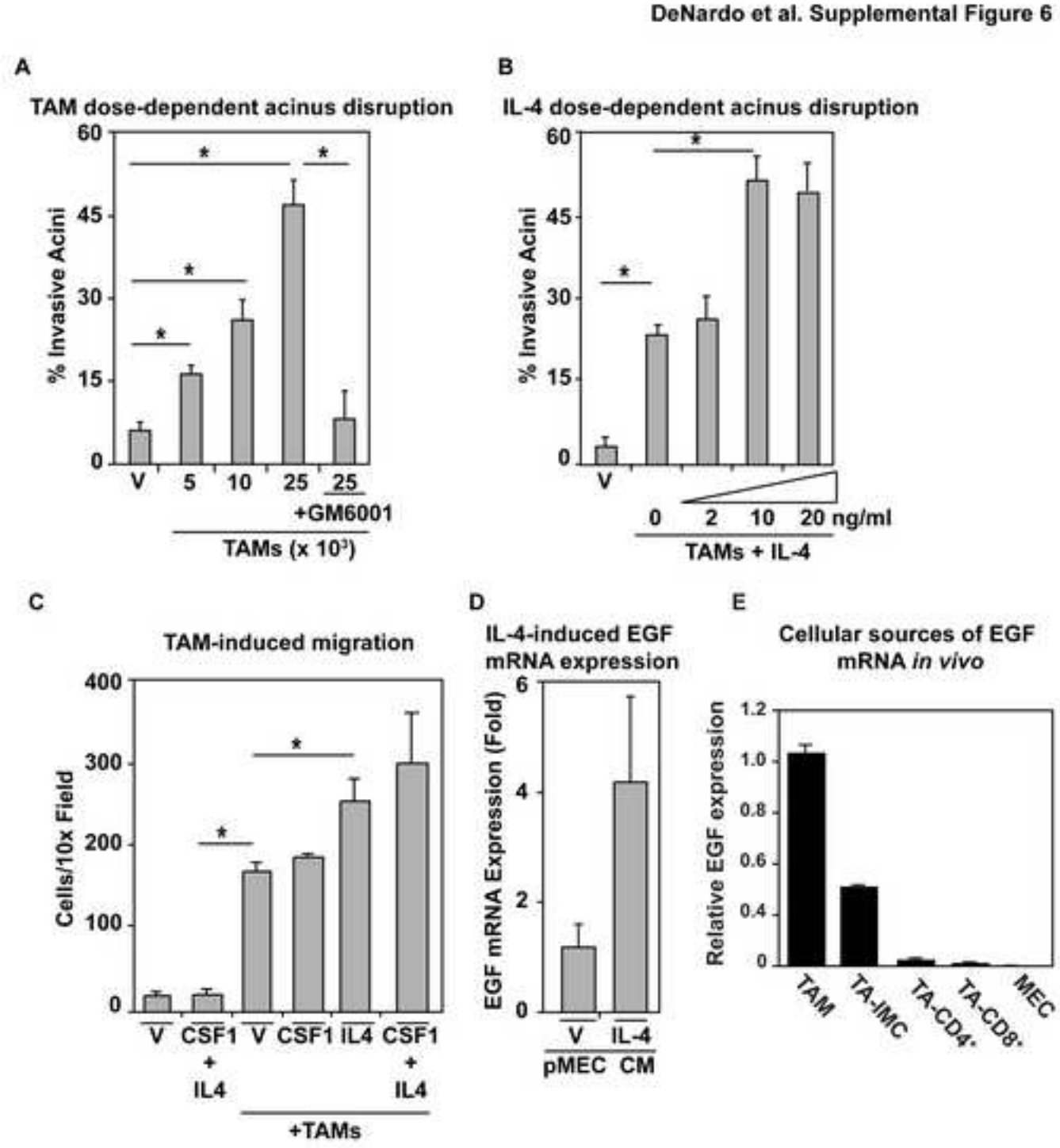

Appendix C

AACR Special Conference, *Chemical and Biological Aspects of Inflammation and Cancer*, Ko Olina Hawai, USA

Inflammation and Cancer: Insights into Organ-specific Regulation of Cancer Development by Leukocytes

Lisa M. Coussens. Department of Pathology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, 513 Parnassus Ave., HSW-450C San Francisco, CA 94143-0502 USA; Phone: 001-415-502-6378; e-mail: Lisa.Coussens@ucsf.edu

The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion is B cell, Ig and FcγR–dependent, during mammary carcinogenesis, T_H2-polarized $CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of M2-polarized macrophages and immature myeloid cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

Funding from the National Institutes of Health and a Department of Defense Era of Hope Scholar Award.

AACR Special Conference in Cancer Research, Tumor Immunology: New Perspectives; Miami FL, USA

Tissue-Specific Mechanisms Regulate Pro-Tumor Immunity

Lisa M. Coussens. Department of Pathology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, 513 Parnassus Ave., HSW-450C San Francisco, CA 94143-0502 USA; Phone: 001-415-502-6378; e-mail: Lisa.Coussens@ucsf.edu

The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular and cellular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate the pro-tumor properties of myeloid cells including their recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis is B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_H2-polarized $CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of M2-polarized macrophages and immature myeloid cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

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6th International Symposium on the Intraductal Approach to Breast Cancer, Santa Monica CA USA

PROTUMOR IMMUNITY AND BREAST CANCER DEVELOPMENT

LISA M. COUSSENS, PH.D.

Department of Pathology and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA 94143, [3]San Francisco State University, San Francisco CA 94132 USA. Email: Lisa.Coussens@ucsf.edu

For decades, it was generally accepted that leukocytic infiltrations in tumors represented a failed attempt of the immune system to eradicate *damaged* cells. While indeed some aspects of failed anti-tumor immunity exist, what we now appreciate is the fact that multiple protumor immune programs are instead co-opted by nascent tumors, and in so doing significantly enhance tumor development, including breast cancer. Based upon our evaluation of human clinical specimens revealing significant infiltration of breast tumor tissue by both T lymphocytes and macrophages, we asked the question as to whether adaptive immunity was perhaps enhancing protumor properties of macrophages and thereby potentiating breast carcinogenesis. Utilizing the MMTV-PyMT mouse model of mammary carcinogenesis, $CD4^+$ T cell-deficient mice, and an *ex vivo* three-dimensional organoid co-culture model, we revealed a tumor-promoting role for T_H2-$CD4^+$ T effector cells that elicit pro-tumor, as opposed to cytotoxic, bioactivities of tumor-associated macrophages (TAMs) and enhancement of pro-metastatic epidermal growth factor (EGF) receptor signaling programs in malignant mammary epithelial cells. These novel findings provide a mechanism explaining how T_H2-activated TAMs achieve HIGH level expression of EGF necessary for inducing survival, invasive growth and metastatic programs in malignant cells, and together indicate that anti-tumor acquired immunity, mediated by $CD4^+$ T lymphocytes are usurped in pro-tumor microenvironments and instead promote cancer by engaging cellular components of the innate immune system, and identifies new cellular targets, namely T_H2-polarized $CD4^+$ T lymphocytes, for anti-cancer therapy.

Funding from the National Institutes of Health and a Department of Defense Era of Hope Scholar Award.

International Cancer Conference, *CANCER 2009*, Dublin IRELAND

PROTUMOR IMMUNITY AND BREAST CANCER DEVELOPMENT

LISA M. COUSSENS, PH.D.

Department of Pathology and Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA 94143, [3]San Francisco State University, San Francisco CA 94132 USA. Email: Lisa.Coussens@ucsf.edu

For decades, it was generally accepted that leukocytic infiltrations in tumors represented a failed attempt of the immune system to eradicate *damaged* cells. While indeed some aspects of failed anti-tumor immunity exist, what we now appreciate is the fact that multiple protumor immune programs are instead co-opted by nascent tumors, and in so doing significantly enhance tumor development, including breast cancer. Based upon our evaluation of human clinical specimens revealing significant infiltration of breast tumor tissue by both T lymphocytes and macrophages, we asked the question as to whether adaptive immunity was perhaps enhancing protumor properties of macrophages and thereby potentiating breast carcinogenesis. Utilizing the MMTV-PyMT mouse model of mammary carcinogenesis, $CD4^+$ T cell-deficient mice, and an *ex vivo* three-dimensional organoid co-culture model, we revealed a tumor-promoting role for T_H2-$CD4^+$ T effector cells that elicit pro-tumor, as opposed to cytotoxic, bioactivities of tumor-associated macrophages (TAMs) and enhancement of pro-metastatic epidermal growth factor (EGF) receptor signaling programs in malignant mammary epithelial cells. These novel findings provide a mechanism explaining how T_H2-activated TAMs achieve HIGH level expression of EGF necessary for inducing survival, invasive growth and metastatic programs in malignant cells, and together indicate that anti-tumor acquired immunity, mediated by $CD4^+$ T lymphocytes are usurped in pro-tumor microenvironments and instead promote cancer by engaging cellular components of the innate immune system, and identifies new cellular targets, namely T_H2-polarized $CD4^+$ T lymphocytes, for anti-cancer therapy.

Funding from the National Institutes of Health and a Department of Defense Era of Hope Scholar Award.

Cancer Research UK Cambridge Research Institute (CRI) Inaugural Annual Symposium, '*Unanswered Questions in the Tumour Microenvironment*', Homerton College, Cambridge UK

Inflammation and Cancer: Insights into Organ-specific Regulation of Cancer Development by Leukocytes

Lisa M. Coussens. Department of Pathology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, 2340 Sutter St., San Francisco, CA 94143 USA; Phone: 001-415-502-6378; e-mail: coussens@cc.ucsf.edu

The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas chronic inflammation of premalignant skin neoplasms is B cell–dependent, during mammary carcinogenesis, T cells appear to play more of a dominant role in regulating pro-tumor and pro-metastatic properties of myeloid cells. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

Funding from the National Institutes of Health and a Department of Defense Era of Hope Scholar Award.

Appendix C

5th Intl Kloster Seeon Meeting on Angiogenesis, Munich GERMANY

Immune Cells as Regulators of Tumor Angiogenesis

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular and cellular mechanisms regulating leukocyte recruitment into neoplastic tissue and once activated, the subsequent role mature leukocytes play as regulators of cancer progression. We have utilized a transgenic mouse model of squamous carcinogenesis as an archetypical model of inflammation-associated neoplastic progression (Coussens et al., 1999; Coussens et al., 2000; Daniel et al., 2003; Junankar et al., 2006). We have reported that activation of humoral immunity in transgenic mice regulates recruitment and activation of myeloid cells into premalignant tissue (de Visser et al., 2005). More recently, we revealed that humoral immune activation of Fcγ receptors on recruited myeloid cells, including mast cells and $CD11b^{+}Gr1^{+}$ cells, functionally regulates cancer development by affecting T lymphocyte function and activation of pro-angiogenic programs in neoplastic tissues. To be presented will be recent insights into organ and tissue-specificity of these adaptive and innate immune cells effector properties, and thoughts on how these properties can be harnessed for effective anticancer therapy.

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21ST Lorne Cancer Conference, Lorne AUSTRALIA

Pro-Tumor Regulation of Cancer Development by Infiltrating Immune Cells

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The concept that leukocytes are components of malignant tumors is not new; however, their functional involvement as promoting forces for tumor progression has only recently been appreciated. We are interested in understanding the molecular mechanisms that regulate leukocyte recruitment into neoplastic tissue and subsequent regulation those leukocytes exert on evolving cancer cells. By studying transgenic mouse models of skin, lung and breast cancer development, we have recently appreciated that adaptive leukocytes differentially regulate myeloid cell recruitment, activation, and behavior, by organ-dependent mechanisms. Thus, whereas premalignant progression, including chronic inflammation, activation of angiogenic programming, tissue remodeling and malignant conversion during skin carcinogenesis is B cell, Ig and FcγR–dependent, during mammary carcinogenesis by contrast, T_H2-polarized $CD4^+$ T cells play a dominant role in regulating pro-tumor and pro-metastatic properties of M2-polarized macrophages and immature myeloid cells, that together regulate metastasis of malignant mammary epithelial cells to lung. To be presented will be recent insights into organ and tissue-specific regulation of epithelial cancer development by adaptive and innate immune cells, and thoughts on how these properties can be harnessed for effective anticancer therapeutics.

Funding from the National Institutes of Health and a Department of Defense Era of Hope Scholar Award.

Appendix C

DOD BCRP Era of Hope Meeting 2008, Symposium Session: *Immune and Inflammatory Contributions to Breast Cancer*, AND *Era of Hope Spotlight Session*, Baltimore MD, USA

$CD4^+$ T Cells Regulate Macrophage Phenotype and Functionally Contribute to Mammary Tumor Development.

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Background and Objectives: During cancer development, the environment in which young 'tumor' cells develop determines their ability to progress to the malignant state. Recent clinical and experimental evidence supports a critical link between chronic inflammation and development of breast cancer. Historically, leukocytes found in and around developing tumors were thought to represent an attempt of the host to eradicate transformed neoplastic cells; however, recent epidemiologic and experimental evidence supports a promoting role for some immune cell types during cancer development. In neoplastic human breast tissue, macrophage, B and T lymphocyte presence increases during progression from pre-malignant in situ to malignant disease reflecting their potential increased significance with regards to malignancy. The objective of our studies is to identify functionally significant immune cell types and/or immune-regulated molecules that potentiate breast carcinogenesis, and determine to what degree breast cancer is susceptible to immuno-modulation as a therapeutic approach.

Methodology: In the present study, we assessed the functional significance of infiltrating adaptive immune cells during development of mammary adenocarcinomas by intercrossing the MMTV-PymT mouse mammary carcinoma model with B and T lymphocyte-deficient mice ($RAG1^{-/-}$), B cell-deficient mice ($JH^{-/-}$) and mice deficient for either or both $CD4^+$ and/or $CD8^+$ T cells.

Results: We found that while loss of B and T lymphocytes did not alter latency of primary tumor development, tumor burden or tumor histopathology, pulmonary metastasis (>80%) and total metastatic tumor burden were significantly ($p < 0.01$) diminished in a $CD4^+$ T lymphocyte-dependent manner. Genetic elimination of $CD4^+$ T cells phenocopied the $PymT/RAG1^{-/-}$ phenotype, as well as correlated with deceased numbers of circulating $PymT^+$ tumor cells and diminished presence of M2 or alternatively activated macrophages present in primary tumors. Using an organotypic 3-dimensional co-culture model with primary $PymT^+$ mammary epithelial cells (MECs) and naïve or tumor associated macrophages and $CD4^+$ T cells, we revealed that tumor-associated $CD4^+$ T cells regulate macrophage behavior/phenotype (polarization), that in turn regulate malignant and invasive behaviors of MECs in an interleukin (IL) 4-dependent manner.

Conclusions: Together, these data indicate that chronic activation of $CD4^+$ T lymphocytes regulates production of type 2 inflammatory cytokines such as IL-4, that in-turn elicits pro-tumor (as opposed to cytotoxic) bioactivities in macrophages that then enhance malignant and metastatic programming of neoplastic mammary tissue.

Appendix C

CANCER RESEARCH UK LECTURE, NCRI Cancer Conference, Birmingham UNITED KINGDOM

Inflammation and Cancer: Insights into Organ-specific Immune Regulation of Cancer Development

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Funding from the National Institutes of Health and a Department of Defense Era of Hope Scholar Award.

University of Michigan, Oral Health Sciences Program and Biomedical Engineering Seminar Series, Ann Arbor, MI USA

Inflammation and Cancer: Insights into Organ-specific Regulation of Cancer Development by Leukocytes

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Appendix C